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SIMPÓSIO INTERNACIONAL SÔBRE VENENOS ANIMAIS

INTERNATIONAL SYMPOSIUM ON ANIMAL VENOMS

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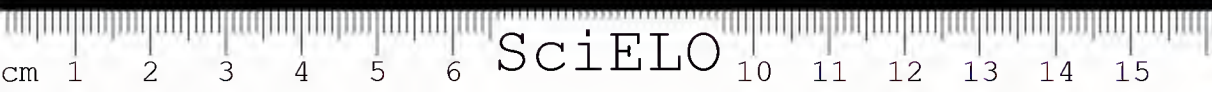
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INTERNATIONAL SYMPOSIUM ON ANIMAL VENOMS

INSTITUTO BUTANTAN

17 a 23 de julho de 1966

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VI

BIOQUÍMICA

BIOCHEMISTRY

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39. ENZYMES OF SNAKE VENOMS AS TOOLS IN BIOCHEMICAL RESEARCH

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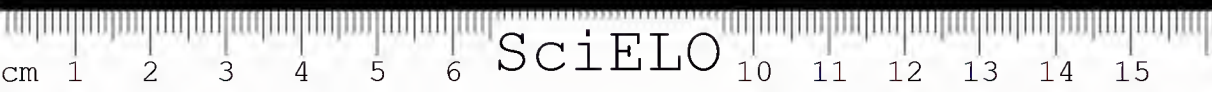
Although biochemists have been slow in recognizing the remarkable properties of snake venoms, they have recently taken so much advantage of this material that only a few typical examples of its use as a tool can be presented here. Some of the reasons for this later development are the following: (a) Snake venoms belong to the most concentrated enzyme sources in nature; (b) their composition is very simple in comparison with a liver homogenate; while only one oxidoreductase is found in snake venoms, it is impossible at the present to say how many occur in the liver cell; (c) they display a remarkable stability when they are properly protected against light and moisture; (d) many excellent preparations of snake venoms are now commercially available; (e) snake venoms tend to attack key substances and key reactions in the animal body; it is, therefore, probably not accidental that we find them of practical value in the study of vitally important metabolic processes.

I have selected three areas to demonstrate the usefulness of snake venoms as a part of analytical and preparative procedures and in the study of certain enzyme mechanisms.

Analytical methods

a. The ophidian L-amino acid oxidase (L-AAO) has been applied to the determination of L-amino acids in D-amino acid preparations. Since the oxidative deamination of L-amino acids proceeds at practically the same velocity in the absence and presence of high D-amino acid concentrations (1, 2), the occurrence of 0.01 per cent of the L-form can be detected in D-amino acid preparations with the help of L-AAO. There exists no more sensitive qualitative or quantitative procedure to test the optical purity of D-amino acids. J. P. Greenstein and his co-workers have sharpened this tool to a high degree (3).

The same enzyme has been applied to the analysis of phenylalanine in the blood and urine of infants suffering from phenylketonuria. This precise, rapid and specific micromethod is based on the measurement of absorption of the enol-borate complex of phenylpyruvic acid generated from the amino acid by the L-AAO of *Crotalus adamanteus* (4, 5). A specimen as small as 0.02 ml of plasma ultrafiltrate is sufficient for the test (6). With appropriate modifications, tyrosine (4, 5), tryptophan (4), and histidine (7) can be determined by the same principle.

Recently, L-AAO has been applied to the identification of L-amino acids separated by paper chromatography. The chromatogram is treated with a venom solution (*C. adamanteus*), phenazine methosulfate, and a tetrazolium compound. The latter acts as an electron acceptor with phenazine methosulfate serving as an electron carrier. As little as 1 μg of those amino acids, which are rapidly deaminated in the presence of the L-AAO, can be recognized by the appearance of formazans (8).

b. L-AAO can be coupled with amino acid releasing enzymes (reaction 1). If the conditions are adequately chosen, the former enzyme becomes the rate limiting

$$\text{Amino acid donor} \xrightarrow{\text{(peptidase)}} \text{L-amino acid} \xrightarrow{\text{(L-AAO)}} \alpha\text{-keto acid} + NH_3 \qquad (1)$$

factor of the system. On this basis, a quantitative method for peptidase activity has been developed (9, 10). Peptides (9, 10, 11), proteins (8), or amino acid esters may be used as amino acid donors; oxidation of the liberated amino acid may then be followed manometrically, photometrically, titrimetrically, or polarographically. Very small activities can thus be determined. If several different amino acids are liberated by the peptidase, these amino acids will compete for the L-AAO. In order to avoid the formation of such complex systems, peptidase systems which release only one amino acid are preferable. The simultaneous production of glycine and L-alanine with other free amino acids does not interfere with the measurement because the former two amino acids display very low affinity for the enzyme and thus can be neglected (3). Amino acid esters serve as excellent substrates because only one amino acid is involved. The lens amino peptidase, in contrast to all other amino peptidases, acts vigorously on esters such as L-phenylalanine ethylester, or L-tryptophan methylester, and can be detected with the help of the L-AAO as shown by Palmberg (12). Recently, this principle has been applied to the determination of peptidase activity of the complex formed between pancreas carboxypeptidase and its antibody (8). The complex, localized with the help of immunoelectrophoresis, is treated with hippuryl-L-arginine and the reagents mentioned above. The peptidase activity of the antigen-antibody complex is recognized by the appearance of the blue formazan.

c. There is a large body of publications dealing with the successful use of phosphodiesterases and pyrophosphatases in the elucidation of the structure of nucleic acids and dinucleotide coenzymes. To single out one recent example, the work of Pfleiderer and Woenckhaus pertaining to the conformation of adenine pyridine dinucleotide (NAD) is briefly summarized here (13). The authors succeeded in separating α-NAD from the β-form of the dinucleotide from yeast NAD by chromatography on Dowex 1 (formate form). In the α-isomer, which is catalytically inactive, the ribosyl residue is connected to the nicotinamide moiety by an α-glycosidic linkage. Cleavage of α-NAD with pyrophosphatase, purified from the venom of *Naja nivea*, resulted in an increase in the ε_{max} at 260 mμ from 17,900 to 19,900. This increase was of the same order of magnitude as previously obtained for the hydrolysis of pure β-NAD by snake venom (14). This was indicative of a similar folding of the molecules and of an interaction between the nicotinamide and adenine rings of both α- and β-forms.

Preparative procedures

a. The work on the enzymatic hydrolysis of α-NAD culminated in the isolation of the α-nicotinamide mononucleotide (αNMN). The topical and chemical properties of the new mononucleotide were thoroughly studied. It differed very little spectroscopically from the β-isomer, but chemically the hydrolysis of the α-isomer was less easily achieved than that of the β-isomer. Without the ophidian enzyme, it would have been difficult indeed to carry out this interesting investigation on the relationship between the chemical structure and certain properties of NAD.

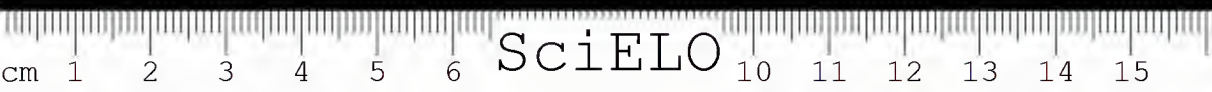
b. Parikh *et al.*, with the help of dialyzed venom of *C. adamanteus*, completely destroyed the L-isomer of the racemic form of more than a dozen aliphatic and aromatic amino acids and isolated the pure D-isomers by alcohol precipitation (15). This method permits the rapid production of certain D-amino acid on a small scale basis. Since the reaction is carried out in the presence of catalase, α-keto acids are formed which remain in the aqueous solution during the precipitation.

c. A similar idea to the resolution of racemic mixtures of amino acids has been successfully applied to the preparation of the first pure native plasmalogen, phosphatidal choline (16). The essential step consisted in treating the crude plasmalogen with ophidian phospholipase A in order to destroy the accompanying lecithins.

d. Since many L-amino acids are quantitatively converted into the corresponding α-keto acids by the combined action of L-AAO and catalase, we have here the basis for a simple and efficient procedure to prepare these compounds as substrates for enzymatic experiments (17). An example is found in the preparation of α-ketoglutaramic acid from glutamine for studies in which the keto acids serve as a receptor in certain transaminase reactions (18).

Mechanisms of enzyme reactions

As a fairly pure and easily accessible flavoprotein, the L-AAO became the object of many studies concerning the mode of action of yellow enzymes.

a. In the very first paper on this enzyme, it was noticed that at high leucine concentration the reaction rate was reduced (1). These results were first explained by J. B. S. Haldane's hypothesis of a substrate having a two-point attachment with the enzyme, the excess substrate competing with the initially bound substrate for the second point (19). Doubts concerning this concept arose when it was found (20) that the phenomenon was more marked with substrates undergoing rapid oxidation in the presence of L-AAO than with homologous substrates sluggishly degraded (Fig. 1) and that the inhibition caused by high substrate concentration decreased with increasing oxygen density (21, 22). Meister and Wellner, on the basis of detailed analysis of the steady state behavior of the enzyme, proposed another interpretation of this phenomenon (23). These authors had succeeded in crystallizing L-AAO from the venom of *C. adamanteus*. The enzyme, with a molecular weight of approximately 130.000, contains two moles of flavin adenine dinucleotide (FAD). The new hypothesis takes into consideration the following two points: Both FAD molecules participate in the

enzymatic process, and each FAD molecule is first converted into a half-reduced state; at high substrate concentration, the FAD is further converted into the fully reduced enzyme. Following this scheme, it would be sufficient to postulate that the half-reduced form is reoxidized more rapidly by molecular oxygen than the fully reduced enzyme. This concept was supported by recently published observations made by Desa and Gilson (24).

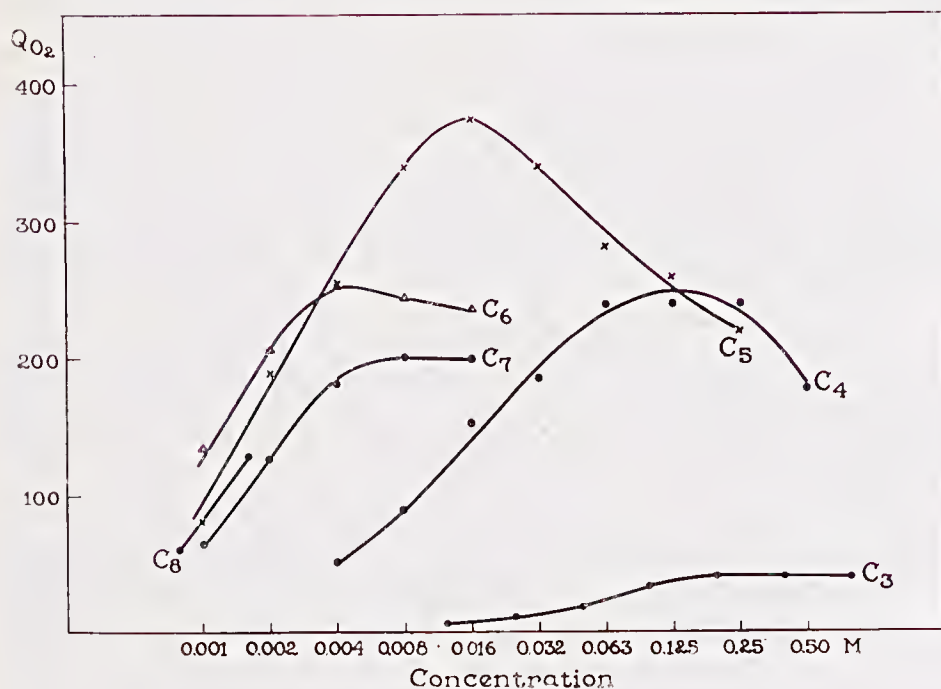



Fig. 1

In order to obtain more information about the simultaneous participation of both FAD molecules in the amino acid degradation and thus about the validity of the hypothesis to explain the inhibition by high substrate concentration, Wellner compared native with reversibly inactivated enzyme (25). The latter was obtained by heating at 38°C for 60 minutes. After the inactivation. no changes in electrophoretic mobility, in sediment coefficients, or in interaction with antibodies were observed. From these observations, it can be concluded that the inactivation is not caused by the loss of FAD, nor by dissociation, aggregation, or denaturation of the enzyme molecule. A shift of the absorption maxima of the FAD, however, indicated an alteration in the mode of binding of the FAD by the protein. These and other observations support the hypothesis that conformational alterations in the environment of the FAD molecules had taken place during the inactivation. The positions of the two flavin moieties relative to each other apparently are changed, thus preventing their concerted action. Although the conclusions are well supported by the data, a direct proof of the simultaneous action of the two FAD molecules remains to be presented. It should be mentioned here that Massey proposed a mechanism which requires the action of only *one* FAD molecule (26).

b. As the last example, it can be seen how studies carried out with L-AAO have led to the introduction of a new concept into the field of enzyme kinetics.

After many years of investigation of the substrate and inhibitor pattern of monoamine oxidase (MAO), we were led to the assumption that some substrates can form two types of complexes with the active site of certain enzymes. Since the substrate pattern of the L-AAO, as known at that time, seemed to indicate the existence of phenomena similar to that encountered in the MAO reaction, and since the L-AAO could be readily crystallized from the venom of *C. adamanteus*, it seemed to be a convenient tool for further analysis.

Our first approach consisted of determining the constants of the Michaelis-Menten relationship for more than 60 different aromatic amino acids and their ring-substituted derivatives (2). Some of data are presented graphically in Fig. 2.

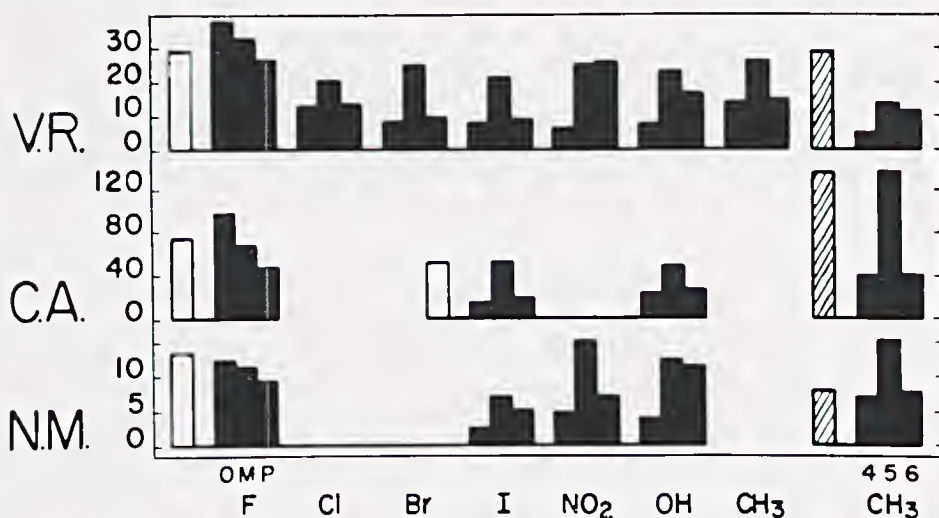



Fig. 2

The maximal velocities for substituted phenylalanines and tryptophans are given for the L-AAO of crude venom of *Vipera russellii*, *C. adamanteus*, and *Naja melanoleuca* as computed from manometric measurements. It can be easily recognized that meta-substitution in phenylalanine and 5-substitution in tryptophan, in general, yield better substrates than other substitutions. It seems as if the *locus*, size, and shape of the substituents rather than their effects on electron distribution affect the maximal rate.

In trying to interpret these and related data, we started from an earlier observation which led us to believe that the aromatic ring of certain substrates of MAO and L-AAO contributes the major part of the binding energy between the active site and the substrate (2). If an aromatic system exists in the active site (27), so the benzene or heterocyclic ring of the substrate can be attached to it, presumably through π-orbitals and hydrophobic bonding. The symmetries of the aromatic rings would allow the formation of more than one complex. One type of complex, called *eutopic* (28) or productive (29), is an intermediate in the conversion of the substrate to the product, while the other type, named *dystopic* or non-productive, is not an intermediate and, therefore, does not lead to

product formation. Three cases may be envisaged: (a) The occurrence of only eutopic complexes, thereby permitting the highest reaction rate of a given enzyme system; (b) the occurrence of dystopic complexes only, thereby preventing substrate degradation; (c) the occurrence of both productive *and* non-productive complexes. Thus, in case (c) the reaction velocity is a function of the ratio of the occurrence of the two types of complexes, this ratio being determined possibly by the shape of the substrate molecules. If we derive in the usual way the relationship between substrate concentration and reaction rate and if we take into consideration the appearance of dystopic complexes, then we arrive at equation II. If no other additional factors

$$v = V \cdot s/[K_e + s\ (1 + K_e/K_d)] \qquad \text{(II)}$$

are involved, then with increasing dystopic character of the enzyme-substrate complex (i.e., as the ratio of occurrence of dystopic to eutopic complexes increases), the Lineweaver-Burk plot is shifted toward increasingly higher ordinate values without a change in slope. Parallel shifts of the Lineweaver-Burk plot have actually been observed on several occasions (2, 28). More general expressions than that given in equation II are found in the papers by Niemann (29) and by Zeller *et al.* (2). Niemann independently conceived the idea of two different types of complexes from his analysis of the substrate pattern of chymotrypsin.

In order to gain further information about the range of applicability of the new concept, we carried out additional experimental investigations. From the outset, we wanted to replace the manometric procedure of measuring L-AAO activity with a more suitable method because the former is fraught with several serious shortcomings. Since the measuring of oxygen consumption with the aid of the oxygen electrode avoids the severest limitations of the manometric method, we adopted the polarographic method. Although the results remained essentially the same, we feel much more secure with our new data.

While the interaction between substrate and flavoprotein is a rather complex process, and the interpretation of the kinetic data leaves many questions unanswered, the binding of an inhibitor by a given enzyme is in general a more straighforward event. We attempted, therefore, to determine whether the same substitution rules are valid for inhibitors as well as substrates. Since ring-substituted benzoic acids block L-AAO competitively, they appeared to be suitable objects for testing this idea. For 8 sets of ortho-, meta- and para- monosubstituted benzoic acids, Clauss determined the concentration which inhibited L-AAO by 50 per cent. In all but one series, the meta-substituted benzoic acids appeared to be the strongest inhibitors. In the series of hydroxylated compounds, the meta- and the para-derivatives were of equal inhibitory power (30).

We summarize our results in the following way. It would seem as if the geometry of the substrate molecule rather than other factors is responsible for the reaction rate. If this idea were correct, the electron density at the reaction center of the substrate would not be important as the rate determining factor. Since a quantitative relationship between electron density, as determined by ring substitution, and reaction rate/k (or reaction equilibria) exists in the form of the Hammett equation (III), this point can be subjected to experimental verification. The constant ρ is characteristic for a given reaction, while the

$$\log k - \log k_o = \sigma \cdot \rho \qquad \text{(III)}$$

constant σ is defined as a measure of the electron density at the reaction center. If, after plotting the left side of equation III against σ, we obtain a straight line, we consider the Hammett relationship as fulfilled. If the slope is positive, a low electron density favors the reaction at the rate determining step, whereas a negative slope indicates that an electron shift toward the reaction center permits the enzymatic process to proceed rapidly. In a number of enzymatic systems, the Hammett equation has been found to describe the relationship between ring substitution and velocity adequately. To test L-AAO in a similar way, Clauss (30) determined polargraphically the reaction rates produced by a number of ring substituted phenylalanines and by the L-AAO from *C. adamanteus*. The data are presented in Fig. 3. The logarithm of the maximal velocity is plotted

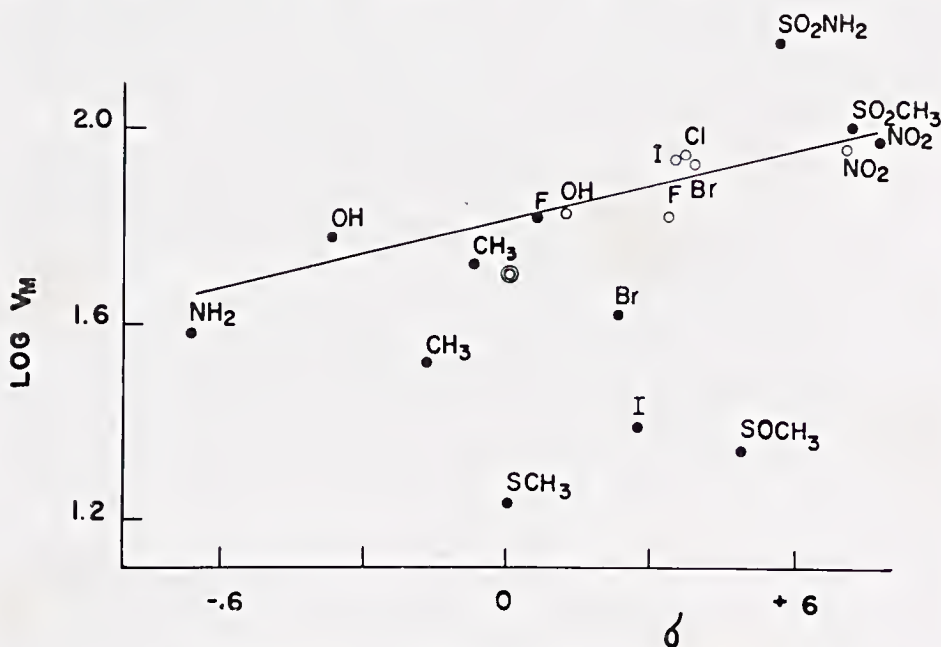



Fig. 3 — Hammet plot for ophidian L-amino acid oxidase (see text).

against σ. While a straight line may be drawn through the points representing several meta-substituted amino acids (open circles), it is obviously impossible to establish a linear relationship for the points representing para-substituted phenylalanines (black circles; the double circle indicates the unsubstituted phenylalanine). This is tantamount to saying that, for the para-substituted phenylalanines, factors other than electron density at the reaction center of the substrate are rate determining. Geometric properties, therefore, may be taken into consideration instead.

If these considerations appear too hypothetical to be presented at this symposium, they may still provide us with a new insight into the biological function of L-AAO. This enzyme originally was thought to play a role in the digestive action of snake venoms (31). The low LD_{50}, as determined for the pure enzyme by Russell *et al.* (32) suggest that it is intimately involved in the toxic action of the venom. This conclusion appears to be untenable in the light of the ex-

periments reported by Zwisler who found that monospecific antisera against L-AAO are not able to reduce the toxicity of the whole venom (33). However, the complex formed by the L-AAO and its antibody has been found to be as active as the free enzyme (25). It seems conceivable that the L-AAO may destroy the local pools of phenylalanine and tyrosine, the precursors of the catecholamines. Since the latter play an important role in the ergotropic reaction of the prey, this removal of the aromatic amino acids should weaken the defense mechanism. From Fig. 1 and from incomplete data presented in several papers (34, 35), one can see high ratios for the maximal velocities of phenylalanine to leucine (V_{phe}/V_{leu}) for elapid venoms as compared with venoms of the other two classes of snakes. If the activity toward leucine appears to be representative of the amount of L-AAO present (2), then low L-AAO concentrations often found in elapid venoms are partially or entirely compensated by a high (V_{phe}/V_{leu}) value. According to our hypothesis, the relative higher power of phenylalanine destruction by elapid venoms is due to a higher degree of eutopic complex formation between L-AAO and aromatic amino acids.

Conclusion

These remarks give only a disappointingly small and by necessity one-sided section of the range of studies carried out with venoms as an enzyme source. But I do hope that they demonstrate the tremendous potential of this tool. Vital Brazil, in whose honor we meet here, used the venoms to overcome their fatal effects. Today, we are able to go beyond Brazil and to use snake venoms for the investigation of biochemical reactions of theoretical and practical importance as testified by many papers presented at this Symposium. In the future, we may add a deeper meaning to the curious fact that the snake in the form of the caduceus is the symbol of the medical profession.

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40. PROTEOLYTIC ENZYMES OF *BOTHROPS* VENOM

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Observations on the proteolytic activity of snake venoms had already been made at the end of the last century. Studies on this interesting biological material, however, developed rather slowly and purification of enzymes from snake venoms was accomplished only recently. The value of proteolytic enzymes as tools in protein chemistry has induced many scientists to search for new proteinases, and a close study of their specificities can only be reached with purified enzymes.

We are presenting the results of purification and properties of three proteolytic enzymes from the venom of *Bothrops jararaca*: one with caseinase activity, a second showing blood-clotting activity and a third with hydrolyzing activity on arginine synthetic substrates. In the crude venom, these activities differ in sensitivity to metal ions, anions and metal-binding agents. Thus, in presence of sulphide and cyanide ions, the caseinase activity is strongly inhibited whereas the benzoylarginine amidase (BAAase) and the blood-clotting activities are unaltered. Calcium ions increase the caseinase activity, have no action on amidase activity and slightly increase the blood-clotting activity when assayed on oxalated plasma. The blood-clotting activity, however, is inhibited in presence of Ca^{2+} ions when assayed on fibrinogen. The caseinase activity is almost completely inhibited by Cd^{2+} ions, the BAAase is slightly decreased, whereas the blood-clotting enzyme is not affected by the same concentration of Cd^{2+} ions (Table I). Also the effect of metal-binding agents is different on these activities (Table II). It can be seen that reduced glutathione inhibits the caseinase and blood-clotting enzymes, and slightly inhibits the BAAase activity of the venom. The caseinase and blood-clotting activities are very much decreased in the presence of ethylenediaminetetra-acetic acid, whereas the BAAase activity is unaffected (1, 2).

The first attempt to purify these enzymes was made by fractional precipitation with ammonium sulphate and showed (1, 2) already a preliminary separation of these activities (Table III). Fraction no. 5 had the strongest activity on casein and gelatin. The protein fraction no. 6 presented the highest blood-clotting activity, showing an amidase activity twice as great as the initial venom; and fraction no. 7 had the highest BAAase specific activity about 5 times greater than that of crude venom and its hydrolytic activity on casein was less than half of the activity presented by the starting material. Simultaneously Holtz and Raudonat (3) reported the separation of two fractions from the venom of *B. jararaca*, by precipitation with ammonium sulphate; one, named "protease",

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had strong proteolytic activity and the other, "koagulin", showed mainly blood-clotting activity with weak proteolytic activity. We further observed that one minute heating in a boiling-water bath was already sufficient to destroy all

TABLE I — EFFECT OF IONS ON PROTEINASE, AMIDASE AND BLOOD-CLOTTING ACTIVITIES OF THE VENOM OF *BOTHROPS JARARACA*

Venom solution was incubated for 1 h. at 35° with test substances (final concn. 2 mM) before addition of substrate. Reaction mixture for the determination of caseinase activity: 1 ml. of venom-ion mixture and 1 ml. of 1% (w/v) casein in 0.05 M-tris buffer, pH 8.8. For amidase activity: 0.2 ml. of venom-ion mixture, 0.4 ml. of 0.05 M-benzoyl-L-arginine amide (BAA) and 0.2 ml. of 0.3 M-tris buffer, pH 8.8. For blood-clotting activity: 0.1 ml. of venom-ion mixture and 0.2 ml. of oxalated horse plasma.

Ion	RELATIVE ACTIVITY Substrate		Clotting time (sec)
	Casein	BAA	
None	100	100	20
Ca^{2+}	136	100	16
Fe^{2+}	94	89	19.8
Mg^{2+}	89	88	19.5
Mn^{2+}	83	100	19.5
Zn^{2+}	46	92	19.5
Co^{2+}	17	86	22.5
Cd^{2+}	15	79	19.2
Hg^{2+}	—	81	39.5
S^{2-}	33	95	21
CN^{-}	59	100	21
F^{-}	100	100	20
$ICH_2\text{-}COO^{-}$	100	100	21

TABLE II — EFFECT OF METAL-BINDING AGENTS ON PROTEINASE, AMIDASE AND BLOOD-CLOTTING ACTIVITIES OF THE VENOM OF *BOTHROPS JARARACA*.

Test substances were incubated for 1 hr. at 35° with venom solution before addition of corresponding substrates. Venom-test substance mixture was incubated with substrate as in Table I.

Added substance	Final concentration (mM)	RELATIVE ACTIVITY			Clotting time (sec)
		Casein	Gelatine	BAA	
None	—	100	100	100	22
DL-Cysteine	50	21	17	—*	—**
Reduced glutathione	50	18	1	42	80
Ethylenediaminetetra-acetic acid	2	22	7	100	240***
8-Hydroxyquinoline	2	13	38	95	—**
Histamine	50	82	67	67	—**

* The effect of cysteine could not be determined because of the presence of L-amino acid oxidase in the venom.
** Not tested.
*** Partially clotted.

TABLE III — COMPARISON OF HYDROLYTIC ACTIVITY ON CASEIN, GELATIN, BENZOYL-L-ARGININE AMIDE AND OXALATED PLASMA OF THE VARIOUS FRACTIONS PRECIPITATED WITH AMMONIUM SULPHATE.

Caseinase specific activity is expressed as the increase in optical density of the trichloroacetic acid filtrate at 275 mμ/mg of protein in the sample. The gelatinase specific activity is expressed as v (flow-time index)/μg of protein in the sample. Benzoyl-L-arginine amidase specific activity is expressed as μg of NH_3 liberated from benzoyl-L-arginine amide/mg of protein in the sample. Specific blood-clotting activity is expressed as 30 (mg. of protein/ml. of the sample that clots the plasma in 30 sec.).

Fraction No.	Saturation with $(NH_4)_2SO_4$	SPECIFIC ACTIVITIES				
		Caseinase		Gelatinase	Amidase	Clotting
		Ca^{2+} ions absent	Ca^{2+} ions present			
1	None	1.53	2.03	3.15	35.6	248
2	0 -0.40	2.09	2.92	2.24	11.3	77.5
3	0.40-0.45	1.33	2.52	2.76	19.7	162
4	0.45-0.50	1.60	2.86	6.28	27.7	219
5	0.50-0.55	1.59	3.43	7.15	33.1	318
6	0.55-0.70	0.61	0.88	1.61	69.0	501
7	0.70-0.80	0.66	0.66	0.94	164.0	U*
8	0.80-1.00	0.34	0.24	0.46	45.0	U*

* Undetectable

caseinase which was activated by Ca^{2+} ions and the amidase specific activity of the venom was not affected by heating up to 5 minutes (Table IV). Meanwhile was verified the presence of a heat-resistant caseinase, which is not affected by Ca^{2+} ions. These results corroborate the finding of Hamberg and Rocha e Silva (4) of a heat-resistant benzoylarginine methyl esterase activity in the venom of *Bothrops jararaca*.

TABLE IV — EFFECT OF HEATING ON THE CASEINASE AND BENZOYL-L-ARGININE AMIDASE SPECIFIC ACTIVITIES OF THE VENOM.

A 2% (w/v) solution of *Bothrops jararaca* venom in saline was heated in a boiling-water bath for different periods. At the end of each period the tube containing the sample was immediately transferred to an ice-cold water bath. Caseinase and benzoyl-L-arginine amidase specific activities are expressed as in Table III.

Time (min.)	SPECIFIC ACTIVITIES		
	Caseinase		Benzoyl arginine amidase
	Ca^{2+} ions absent	Ca^{2+} ions present	
0	1.54	2.06	45.2
0.5	0.74	1.11	41.8
1	0.43	0.44	54.2
2	0.44	0.41	55.4
3	0.47	0.47	54.3
5	0.44	0.46	53.1
10	0.36	—	39.3
20	0.39	—	27.1

It was found that heating the venom in presence of cacodylate buffer up to 63° destroyed 75 per cent of the blood-clotting activity and 50 per cent of the caseinase activity. So, the heat treatment further distinguishes the amidase from caseinase and blood-clotting activities.

When the amidase fraction, partially purified by heating followed by precipitation between 70 and 80 per cent saturation with ammonium sulphate, free from caseinase activity and still presenting a low blood-clotting activity, was analysed by zone electrophoresis on starch column, evidence was given of two independent entities (Fig. 1). These activities were separated into two distinguishable peaks (5).

Habermann (6), by fractional precipitation with acetone followed by paper electrophoresis, showed a blood-clotting fraction in the *B. jararaca* venom.

The blood-clotting enzyme was purified by Fichman and Henriques (7) from the crude venom by step-wise elution after chromatography on a DEAE-cellulose column. The peak of blood-clotting activity was eluted with 0.3 M sodium caco-
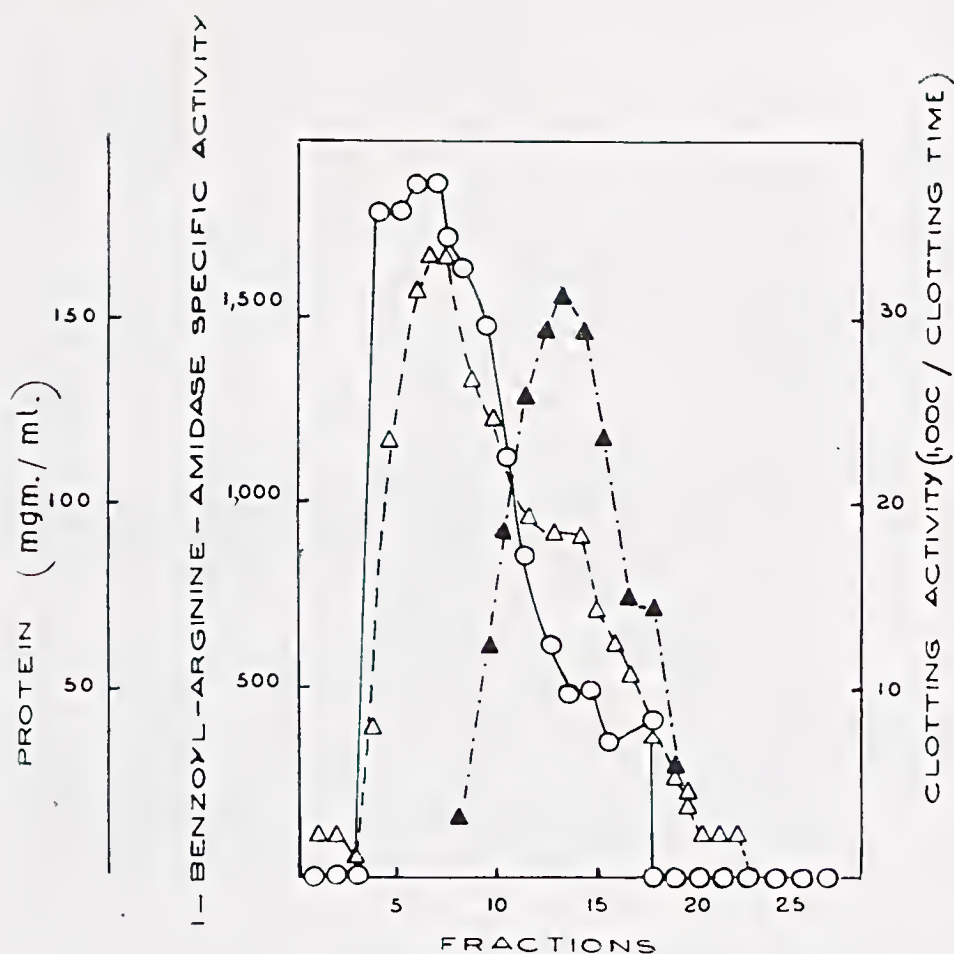
Fig. 1 — Electrophoresis on a starch column of a crude preparation of *Bothrops* protease A (protein fraction precipitated between 70 and 80 per cent saturation with ammonium sulphate). Tris buffer, pH 7.2, I 0.05. △, protein concentration; O, benzoyl-L-arginine amidase specific activity; ▲, blood-clotting activity.

dylate buffer, pH 6.5 (Fig. 2). After rechromatography of this fraction under the same conditions, a protein peak was obtained which showed a blood-clotting activity 9 to 10 times higher than that of the crude venom, an amidase activity about three times greater than that of the starting material. and no caseinase activity. The yield of this partially purified enzyme corresponded to 26 per cent of the total blood-clotting activity of the venom (7). This partially purified blood-clotting enzyme liberates a peptide from fibrinogen (8). This fact, also reported by Blombäck and Vestermark (9). shows that the blood-clotting activity is due to the proteolytic action of the enzyme. When the partially purified blood-clotting enzyme was compared to thrombin, it showed a potency corresponding to approximately 153 NIH thrombin units/mg of protein enzyme (7).
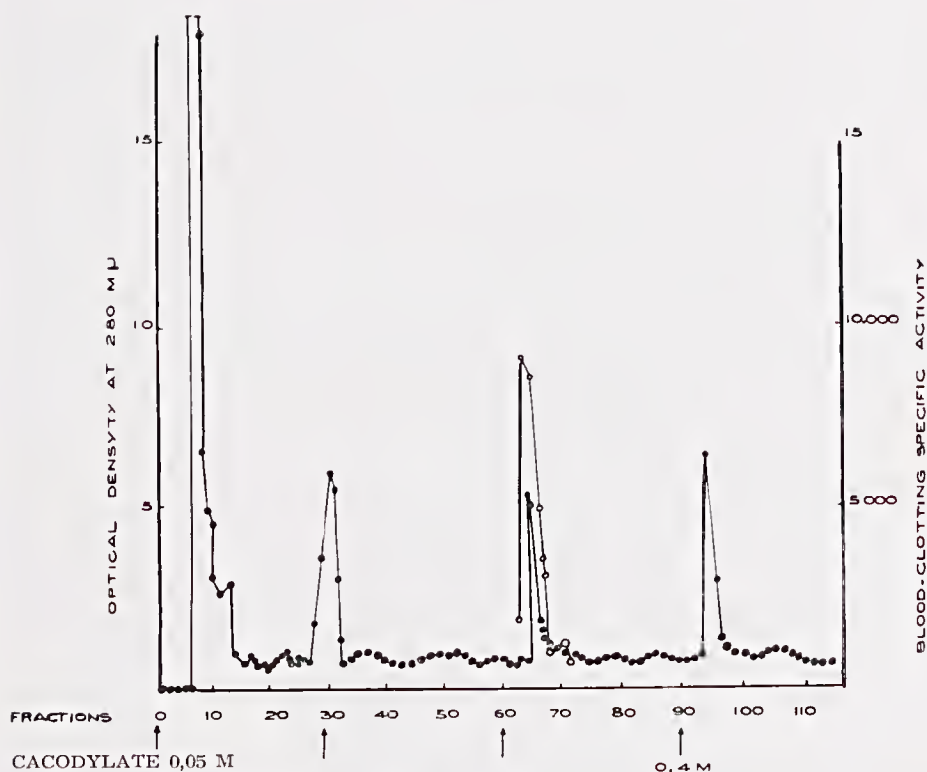
Fig. 2 — Chromatography of venom of *Bothrops jararaca* on DEAE-cellulose column. ●, protein concentration (optical density at 280 mμ); O, blood-clotting specific activity.

The heat-resistant enzyme, which hydrolyzes benzolarginine amide and was demonstrated to be different from the blood-clotting enzyme, was purified by chromatography on a DEAE-cellulose column. The enzyme was eluted with 0.3 M Tris-HCl buffer, pH 7.6 and was completely free from the blood-clotting enzyme, which was eluted with 0.2 M Tris-HCl buffer (Fig. 3). The highest BAAase and TAMEase (toluenesulfonyl methyl esterase) activities were found in the fourth eluted peak (10). This enzyme, denominated *Bothrops* protease A, when re-chromatographed under the same conditions reached a BAAase specific activity 68 times as great as that of the crude extract, with a corresponding 6.3 per cent yield of the total BAAase activity of the starting venom. The capacity of the eluted fractions to liberate kinin (11) was also studied. The peak of *Bothrops* protease A had about the same kinin-releasing activity as the starting heated venom. This finding shows that the enzyme *Bothrops* protease A, having the highest hydrolyzing activity on arginine synthetic substrates, is independent from the kinin liberating enzyme.

The TAMEase as well as the BAAase activities of *Bothrops* protease A are not inhibited by trypsin inhibitors as ovomucoid and soybean inhibitor, on agreement with the observations made by Deutsch and Diniz on the crude venom (12), while diisopropylfluorophosphate inhibits trypsin and *Bothrops*
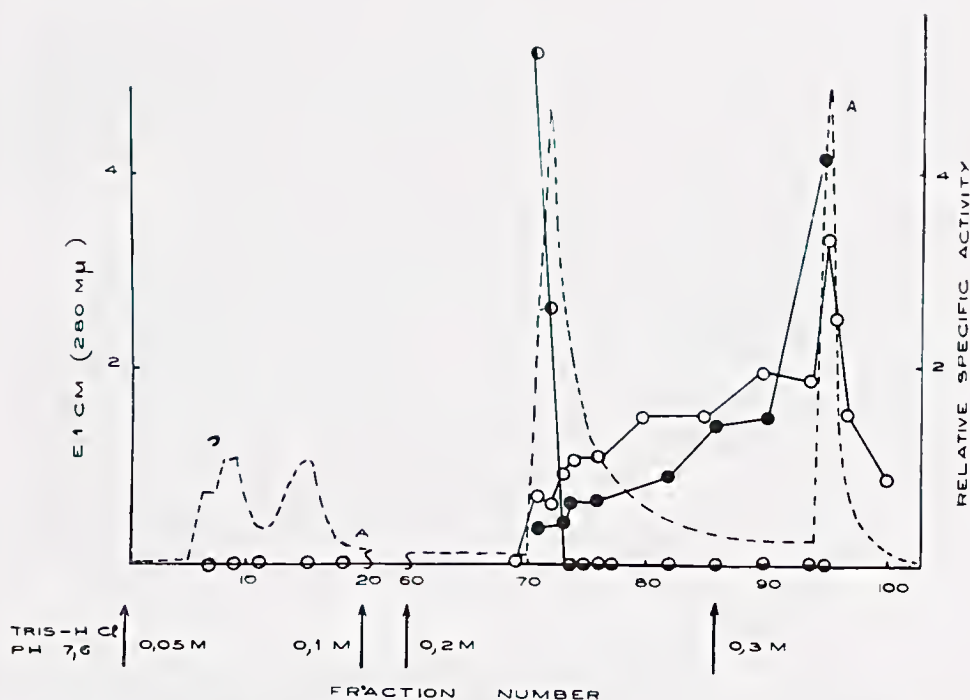
Fig. 3 — Chromatography on DEAE-cellulose column of *Bothrops* protease A precipitated between 70 and 80 per cent saturation with ammonium sulphate. Step-wise gradient elution with tris buffer, pH 7.6. The broken line A presents the extinction of fractions (vol. 20 ml) at 280 mμ (1 cm light path); O, relative specific benzoyl-L-arginine amidase activity; ●, relative specific p-toluene sulfonyl-L-arginine methyl esterase activity; ◐, relative specific blood-clotting activity.

protease A. The similarity between this enzyme and trypsin, when hydrolyzing arginine synthetic substrates, lead us to compare the hydrolytic power of *Bothrops* protease A to twice crystallized trypsin on several synthetic substrates as BAA, TAME, LEE (lysine ethyl ester) and protamine. The relative potency of *Bothrops* protease A on these substrates was found to be, respectively, one eighth, one half, one hundredth and one ninth as great as that of twice crystallized trypsin (Table V).

Trypsin, as it is well known, is unstable at its pH optimum. By contrast, *Bothrops* protease A is not affected by hydrogen ion concentration. In Table VI results concerning the stability (at room temperature) of *Bothrops* protease A, between pH 3 and 9 are shown. There was no decay of BAAase or TAMEase activities measured, up to 26 hours (10).

To confirm the previous observations on the hydrolysis of synthetic substrates and also to characterize the proteolytic activity of *Bothrops* protease A on a peptide chain, the action of this enzyme on insulin and on its B chain was studied. Four peptides were obtained by the hydrolytic activity of *Bothrops* protease A on the B chain of oxidized insulin, which were separated by high-voltage paper electrophoresis at pH 3.9 (Fig. 4), and denominated

TABLE V — COMPARISON OF HYDROLYTIC ACTIVITIES OF TRYPSIN AND *BOTHROPS* PROTEASE A ON SYNTHETIC SUBSTRATES AND PROTAMINE

Benzoyl-L-arginine amidase specific activity is expressed as μmoles of BAA hydrolyzed/mg. of protein enzyme in the sample. Toluenesulfonyl-L-arginine methyl esterase specific activity is expressed as μmoles of TAME hydrolyzed/min./mg. of protein enzyme. L-lysine ethyl esterase specific activity is expressed as μmoles of L-lysine ethyl ester hydrolyzed/min./mg. of protein enzyme in the sample. Protaminase specific activity is expressed as μg of free-amino-N_2 liberated/min./mg. of protein enzyme in the sample.

Hydrolytic activity on	SPECIFIC ACTIVITIES	
	Trypsin	*Bothrops* protease A
Benzoyl-L-arginine amide	9434	1118
p-Toluenesulfonyl-L-arginine methyl ester	504	230
L-Lysine ethyl ester	19	0.18
Protamine	2260	254

TABLE VI — THE pH EFFECT ON THE STABILITY OF *BOTHROPS* PROTEASE A.

Each reaction mixture kept at 25° contained 1 ml. of enzyme solution in saline and 1 ml. of one of the following solutions: 0.005 M-tris buffer, pH 9.0; 0.005 M-tris buffer, pH 8.0; 0.002 N-HCl, 0.2 N-HCl and 2 N-HCl, to give, respectively, the following pH values: 9.0, 8.0, 3.0, 1.0 and 0.2. Samples of 0.2 ml. were withdrawn at the end of 0, 2, and 26 hours. Benzoyl-L-arginine amidase and toluene sulfonyl-L-arginine esterase specific activities are expressed as described in Table III and Table V, respectively.

pH	Time (hours)	SPECIFIC ACTIVITIES	
		TAMEase	BAAase
	0	38	930
9.0	2	40	880
	26	35	850
	0	35	930
8.0	2	43	840
	26	38	790
	0	36	880
3.0	2	39	880
	26	39	820
	0	29	830
1.0	2	23	740
	26	27	690
	0	29	700
≃ 0.2 (1N-HCl)	1.5	19	690
	26	19	480

peptides 0, 1, 2 and 3. Peptide 0 remained on the initial line and peptide 1, 2 and 3 moved towards the cathode. *Bothrops* protease A has no hydrolytic action on the A chain of insulin (13), a conclusion supported by the demonstration that the same three peptides moving to the cathode were observed on high voltage paper electrophoresis of incubating mixture of *Bothrops* protease A with oxidized insulin or with its B chain (Fig. 5).
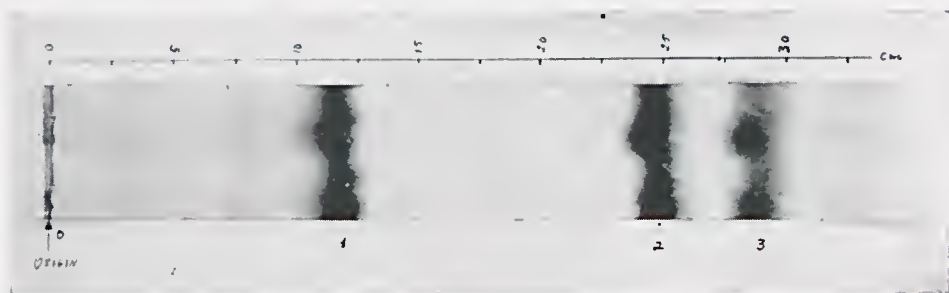
Fig. 4 — Paper electrophoresis of oxidized B chain of insulin incubated with *Bothrops* protease A. Pyridine-acetic acid-water buffer, pH 3.9. Electrophoresis for 4 h. at 1000 V. The direction of migration is from left to right towards the cathode.
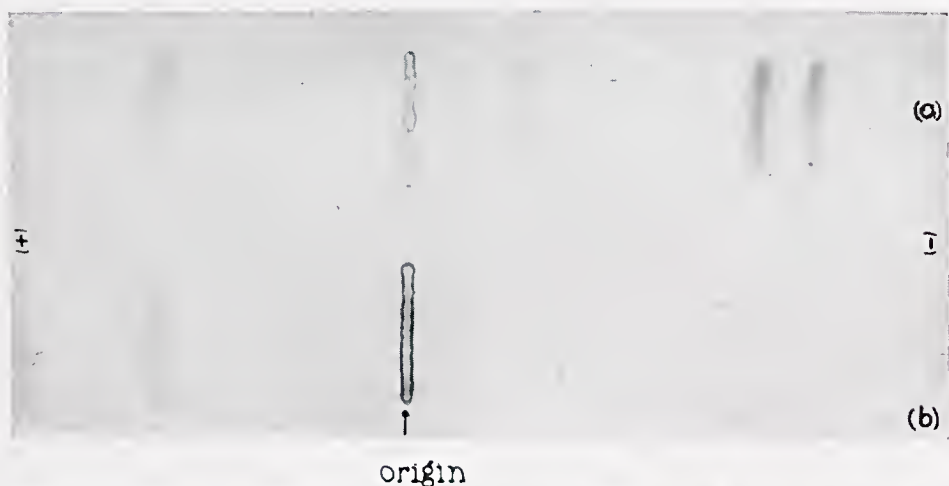
Fig. 5 — Paper electrophoresis of oxidized insulin incubated with *Bothrops* protease A. Pyridine-acetic acid-water buffer, pH 3.9. Electrophoresis for 2.30 h. at 1000 V. (a). Oxidized insulin as control (b).

Investigations of the amino acid composition and N-terminal amino acids of these peptide fragments are summarized in Table VII. Unless proline, lysine and threonine, all the other amino acids of the oxidized B chain of insulin were found by acid hydrolysis of peptide 0. Since it contained only DNP-phenylalanine as N-terminal amino acid, it can be concluded that this peptide resulted from hydrolysis between amino acids 22 (arginine) and 23 (glycine). Glycine and phenylalanine were the two amino acids found in peptide 1, and the DNP-derivative of this peptide proved to be DNP-glycine. After dinitrophenilation,

TABLE VII — AMINO ACID COMPOSITION OF PEPTIDES OBTAINED BY THE ACTION OF *BOTHROPS* PROTEASE A ON OXIDIZED B CHAIN OF INSULIN

Peptide	Amino acids	DNP-amino acid ether-soluble
0	All found in oxidized B chain of insulin excepting Pro, Lys and Thr	DNP — Phe
1	Gly, Phe	DNP — Gly
2	Lys, Thr, Ala, Tyr, Tyr X, Pro	di DNP — Tyr
3	Lys, Thr, Ala, Pro	DNP — Thr

tyrosine was found as the N-terminal amino acid of peptide 2. By acid hydrolysis it turned out to be a pentapeptide of threonine, proline, lysine, alanine and tyrosine (as well as a small amount of tyrosine X, the formation of which could not be entirely prevented). Consequently peptide 2 must have been formed due to the hydrolysis between amino acids 25 (phenylalanine) and 26 (tyrosine) of the B chain of oxidized insulin. The fastest electrophoretic moving peptide (peptide 3) contained the four last amino acids (Thr, Pro, Lys and Ala) of B chain of insulin and DNP-Thr was made evident as its DNP-derivative.

It can be concluded from these data that the sites of action of *Bothrops* protease A on oxidized B chain are those indicated in Fig. 6.

B CHAIN OF OXIDIZED INSULIN

Phe—Val—Asp—Glu—His—Leu— $CySO_3H$—Gly—Ser—His—Leu—Val—Glu—Ala—Leu—
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

—Tyr—Leu—Val—$CySO_3H$—Gly—Glu—Arg—Gly—Phe—Phe—Tyr—Thr—Pro—Lys—Ala
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
↑ (22–23) ↑ (25–26) ↑ (26–27)
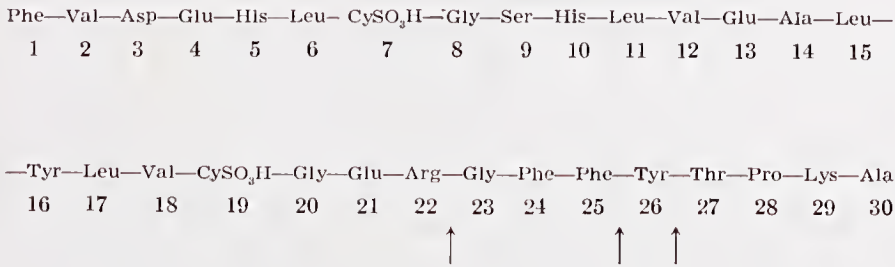
Fig. 6 — Summary of the specificity of *Bothrops* protease A on oxidized B chain of insulin. The arrows indicate the sites split by the enzyme.

The action of *Bothrops* protease A on tyrosine bonds must be associated to a very special peptide configuration, as only the 26-27 tyrosine bond (Tyr-Thr) was split. The other tyrosine bonds, 16-17 (Tyr-Leu) in B chain as well as the 14-15 (Tyr-Glu) and 19-20 (Tyr-$CysSO_3H$) in A chain, were not hydrolyzed. This selective action is also shown towards the basic amino acid bonds, 22-23 (Arg-Gly) and 29-30 (Lys-Ala); only Arg-Gly bond was split by the enzyme and confirms the previous results on lysine and arginine synthetic substrates.

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Discussion

T. Suzuki: "It is interesting that *Bothrops* protease A differs completely from the three proteinases of *A. halys blomhoffii* venom in their hydrolytic actions on insulin B chain".

F. R. Mandelbaum: "In fact, the proteinases purified from the *A. blomhoffii* venom hydrolyze mostly peptide bonds involving the amino groups of leucine and phenylalanine and the enzyme *Bothrops* protease A has no activity on these bonds".

H. Michl: "Are the proteolytic enzymes of *B. jararaca* venom serin-enzymes?"

F. R. Mandelbaum: "Yes, as trypsin, *Bothrops* protease A is inhibited by diisopropylfluorophosphate (DFP), however, the complete inhibition of that enzyme is only reached with a 10 times higher concentration of DFP".

Mem. Inst. Butantan
Simp. Internac.
33(2):371-378, 1966

41. ENZYMES ET TOXINES DES VENINS DE SERPENTS. RECHERCHES BIOCHIMIQUES ET IMMUNOLOGIQUES SUR LE VENIN DE *NAJA NIGRICOLLIS*

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Cet exposé ne constitue pas une revue des recherches publiées sur les enzymes et les toxines des venins de serpents. Une telle entreprise dépasserait les limites d'un bref entretien en raison du nombre considérable des publications parues sur ce sujet (1, 2). Notre but est de vous présenter les résultats d'expériences récentes entreprises dans le dessein d'établir une relation de causalité entre les antigènes décelés dans un venin par les méthodes d'immuno-diffusion et d'immuno-électrophorèse et les facteurs enzymatiques et toxiques de ce même venin.

Disposant de quantités relativement importantes de venin de *Naja nigricollis*, un ELAPIDAE africain, nos recherches on été orientées vers l'étude de ce poison.

D'une manière générale, on constate que les propriétés nocives des venins d'ELAPIDAE et celles des venins de VIPERIDAE ou de CROTALIDAE different. L'expérience nous enseigne, par exemple que la plupart des venins neurotoxiques des ELAPIDAE d'Afrique contiennent peu ou ne contiennent pas d'enzymes protéolytiques, alors que ceux des CROTALIDAE américains, objet de recherches approfondies de la part des expérimentateurs brésiliens, dégradent les protéines.

Notre choix étant dicté par les seules raisons matérielles, nous avons procédé de la manière que voici.

MÉTHODES

Dans des expériences préliminaires, le venin de *Naja nigricollis* est filtré à travers un gel de dextrane, le "Sephadex G_{100} fine" choisi en raison de ses propriétés d'exclusion. Six fractions sont obtenues par ce procédé. Elles sont lyophilisées et conservées à basse température.

La dernière fraction ou "fraction VI" contient un facteur d'une grande nocivité, que nous appelons "toxine α" (3) et des protéines de masses moléculaires voisines. Pour purifier cette toxine α, la fraction VI a été de nouveau filtrée à travers un gel de "Sephadex" plus sélectif, le "Sephadex G_{50} fine". Les techniques de ces filtrations sont décrites "in extenso" dans une précédente publication (4).

Chaque fraction est soumise à une analyse immunologique suivant les méthodes d'immuno-diffusion double en milieu gélifié (5) et d'immuno-électrophorèse (6). Par ce dernier procédé on obtient la séparation des antigènes contenus dans des échantillons de 5 à 10 μg des produits examinés. Un courant de 1,5 v/cm est maintenu pendant 2 heures et le tampon utilisé est la solution usuelle de véronal sodique/HCl 0,025 M (pH 8,2). Dans les deux séries d'expériences on précipite les antigènes par l'immuno-sérum 984 qui provient d'un cheval hyperimmunisé par des injections répétées de venin de *Naja nigricollis.*

Résultats

1 — Le tracé de la figure 1, illustre le résultat de la filtration de 500 mg de venin de *Naja nigricollis* à travers le "Sephadex G_{100} fine". Il est défini par une mesure absorptiométrique de la teneur en protéines de chaque échantillon de filtrat recueilli (λ = 2.800 Å). Ces échantillons sont répartis en 6 lots correspondant aux fractions I à VI.
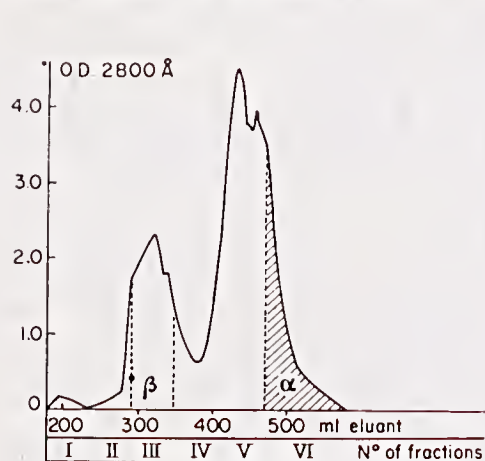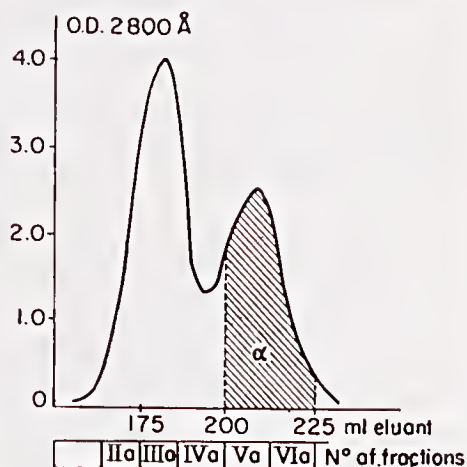
Fig. 1 Fig. 2

Le tracé de la figure 2 correspond à la filtration de la fraction VI à travers le "Sephadex G_{50} fine". Les sous-fractions Va et VIa ainsi obtenues sont définies par le second pic de ce graphique. Soumis à une filtration de contrôle sur "Sephadex G_{50} fine", le produit terminal semble homogène.

2 — La figure 3 représente une préparation dans laquelle 25 μg de venin de *Naja nigricollis* ont été analysés par immuno-électrophorèse et à titre comparatif, la représentation schématique des antigènes dissociées par le même procédé.

Chaque antigène précipité étant désigné par un symbole, on constate que l'antigène correspondant au premier arc *a* se déplace vers l'anode. Il apparaît seulement dans les préparations les plus riches en venin. Un second antigène que nous désignons par la lettre A est sensiblement iso-électrique à pH 8,2. Plusieurs arcs constituent ensuite le groupe des antigènes B. Peu distincts dans le immuno-électrophorèses du venin total, ils ont été l'objet d'une analyse approfondie dont les résultats sont exposés dans les paragraphes réservés aux fractions I et III.

Serum anti-naja nigricollis 0,2 ml

a b

+ A 25 γ B B B C D_1 D_2 D_3 —
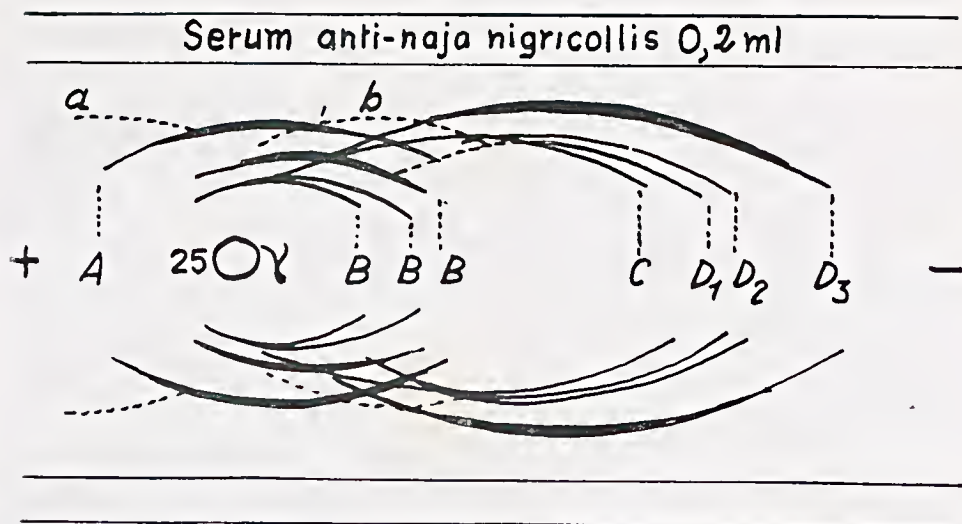
Fig. 3

Un antigène mal défini, *b* est observé dans les préparations qui contiennent une forte quantité de venin.

L'arc de précipité C correspond à un antigène basique. Cet arc est caractérisé par son amplitude et son aplatissement. Enfin, trois antigènes cathodiques précipitent selon les arcs D_1, D_2 et D_3. Onze parmi les treize antigènes dénombrés sont répartis dans les fractions I, III et VI.

La fraction I est essentiellement composée de 4 antigènes du groupe B et la fraction III contient l'antigène A et 3 antigènes du même groupe B.

Les expériences d'immuno-diffusion selon Ouchterlony démontrent que parmi ces antigènes un seul est commun aux fractions I et III. Cette observation est confirmé par l'expérience que voici. Lorsqu'on fait réagir suivant la même technique la fraction III sur un échantillon de sérum 984 épuisé par la fraction I ou la fraction I sur un échantillon du même sérum épuisé par la fraction III, trois lignes de précipité se forment dans chaque préparation. Au contraire le sérum 984 tel quel fait apparaître quatre lignes de précipité dans les préparations témoins. Cette observation nous a conduit à adopter la nomenclature suivante pour définir les antigènes du groupe B.

$$\text{Fraction I} \begin{cases} 1B_1 \\ 1B_2 \\ 1B_3 \\ 1B_4 \end{cases}$$

$$1B_2 = 3B_2 = B_2$$

$$\text{Fraction III} \begin{cases} 3B_1 \\ 3B_2 \\ 3B_3 \\ A \end{cases}$$

Les antigènes $1B_2$ et $3B_2$ étant identiques, nous les désignons par le symbole B_2.

La fraction VI comprend les antigènes fortement basiques C, D_1, D_2 et D_3. Quant aux fractions II, IV et V, elles se comportent comme des mélanges des fractions voisines. Dans la fraction IV, cependant, sont associés aux antigènes A et C les deux antigènes *a* et *b* dont la proportion semble faible dans le venin tel quel.

La fraction α, obtenue par deux filtrations successives de la fraction VI à travers le "Sephadex G_{50}" précipite suivant un arc correspondant à D_3 lorsqu'on la soumet, à la dose de 25 à 50 μ à l'immuno-électrophorèse. Dans les mêmes conditions, cependant, des doses plus fortes de cette fraction soit 100 à 500 μg, non seulement font apparaître l'arc de précipitation D_3, mais encore des arcs correspondant à D_2, D_1 et C dont la position au voisinage de l'axe de la préparation indique que ces antigènes sont en faible proportion.

Une étude de l'action dénaturante de la chaleur sur les antigènes qui viennent d'être énumérés complète ces observations. A cet effet, on chauffe à 96° dans un bain-marie (2) une solution contenant 10 mg de venin par cm^3 d'eau pure * et on prélève, à intervalles réguliers, des échantillons de cette solution qui sont aussitôt soumis à l'épreuve de l'immuno-électrophorèse.

Dans ces conditions en moins d'un minute l'antigène $1B_4$ est privé de sa propriété de précipiter en présence de l'anticorps spécifique.

A l'exception de l'antigène B_2, les autres antigènes du groupe B ne précipitent plus après 5 minutes. Les délais de la perte de leur pouvoir de précipiter sont de 20 minutes pour l'antigène A, de 30 minutes pour l'antigène D_2, de 45 minutes pour l'antigène C, de 55 minutes pour l'antigène B_2 et de 60 minutes pour l'antigène D_3.

Parallèlement à ces recherches, nous avons entrepris de définir les propriétés enzymatiques et toxiques du venin de *Naja nigricollis* et à titre comparatif, celles des fractions obtenues à partir de ce venin.

Les techniques utilisées dans ces essais sont rapportées dans une publication séparée (4).

Si on admet que la dissociation des protéines par la méthode de filtration sur les "Sephadex" est fondée sur leur exclusion dans l'ordre décroissant de leurs masses moléculaires, on est conduit à penser que la fraction I est constituée par les protéines du venin dont la masse moléculaire est plus élevée.

Cette fraction I brise les liaisons carboxy-ester de l'acetylcholine mais elle est sans effet sur les acides aminés estérifiés. Une corrélation a été établie par Hamberg et Rocha e Silva (7) entre l'aptitude que possède le venin de *Bothrops jararaca* à hydrolyser le B.A.E.E et sa propriété de libérer de la bradykinine dans l'organisme animal.

Dans cet ordre de fait, ni le venin de *Naja nigricollis* ni aucune fraction examinée n'ont une action sur le B.A.E.E, le B.A.M.E, le T.A.M.E et l'A.T.E.E.

La même fraction I hydrolyse la liaison peptidique de la glycyl-leucine, et une des deux liaisons de la glycyl-glycyl-glycine et de la glycyl-leucyl-tyrosine mais elle est sans effet sur l'hémoglobine et la caséine.

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* Eau desionisée.

Au contraire, elle rompt très aisément les liaisons phospho-ester du phosphate de paranitrophenol, de l'AMP. du bi-phosphate de paranitrophenol, de l'ADP et de l'ATP.

Son action dépolymérisante à l'égard de l'acide hyaluronique est faible.

Elle possède, enfin, la propriété de provoquer *in vitro* la coagulation du sang.

La fraction III contient non seulement la phospholipase A productrice de lysocithine aux dépens de la lécithine mais encore un facteur lytique direct. Elle détruit le complément du sérum de cobaye, diminue la vitesse de la sédimentation globulaire, dépolymérise l'acide hyaluronique et inhibe le phénomène de la coagulation. Enfin, cette fraction III est légèrement toxique, sa Ld_{50} pour la souris de 18-20 g, éprouvée par la voie veineuse est de 16,31 μg alors que dans les mêmes conditions celle du venin est de 12,36 μg.

La fraction VI exerce une action anticholinestérasique sur l'acétyl-cholinestérase des globules rouges du cheval mais elle est sans effet sur la cholinestérase que nous avons décelée dans la fraction I.

D'autre part, cette fraction VI est toxique et provoque des paralysies chez la souris et le lapin.

Le produit le plus pur que nous avons obtenu après deux filtrations de cette fraction à travers un gel de "Sephadex G_{50}", exerce encore une action anticholinestérasique mais sa Ld_{50} pour la souris est faible. Elle est de 2 μg dans les conditions qui viennent d'être définies. Nous l'avons désignée sous le nom de toxine α, et nous avons conclu d'une étude préliminaire (4) que cette toxine est une protéine de faible poids moléculaire. Elle franchit, en effet, les membranes d'acétate de cellulose et son coefficient de sédimentation exprimé en unités Svedberg est 0,84 (centrifugeuse Spinco; cellule standard de 12 m/m. angle 2°; concentration du produit 10 mg/cm³; vitesse de rotation 59.780 t/m; temps de centrifugation 96 min; température ambiante 20° ± 0,1°).

Une première analyse de ses acides aminés révèle qu'elle contient de fortes proportions d'arginine, de glycine, de lysine, d'acide aspartique, d'acide glutamique et de threonine (3).

Une collaboration a été établie avec J. Porath, E. Karlsson et D. Eaker de l'Université d'Uppsala qui ont bien voulu consacrer une partie de leur activité à l'étude de la toxine α et entreprendre une analyse de la structure de ce polypeptide.

Ces résultats acquis, il importait d'établir une relation entre les antigènes définis par les méthodes immunologiques fondées sur la précipitation mutuelle des antigènes et des anticorps dans un gel et les diverses propriétés qui viennent d'être énumérées.

Des globules de cheval additionnés de lécithine puis versés à la surface d'une préparation de la fraction I ou du venin tel quel, soumis au préalable à une immuno-électrophorèse, sont lysés entre les arcs de précipité de l'antigène A qui peut ainsi être considéré comme étant la phospholipase. En l'absence de lécithine et de la solution tamponnée dont le phosphate se comporte comme un agent inhibiteur, on observe une hémolyse entre les arcs de l'antigène précipité $3B_3$, homologue vraisemblablement du facteur lytique direct décrit par Habermann et Neumann (8) à propos d'autres venins.

L'emploi de la technique d'Uriel (9) adaptée à l'hydrolyse des dérivés phosphorés du nitrophenol permet de suivre l'action des six fractions sur les liaisons

phospho-ester. L'hydrolyse du biphosphate de paranitrophénol se développe entre les arcs de précipité de l'antigène $1B_1$.

Les expériences sur la thermo-sensibilité des divers antigènes qui viennent d'être dénombrés nous enseignent qu'à la température de 96° l'antigène $1B_4$ perd en quelques secondes son aptitude à former un précipité au contact de l'anticorps spécifique. Or dans les mêmes conditions la fraction I perd également sa propriété de coaguler le sang. Cette observation incite à identifier l'antigène thermolabile $1B_4$ facteur accélérateur de la coagulation sanguine. Privée de ce facteur, la même fraction I exerce, au contraire, une action anticoagulante. On est conduit à conclure, d'une part, que dans les conditions habituelles d'expérimentation le facteur coagulant de la fraction I exerce une action dominante, et, d'autre part, que les propriétés anticoagulantes communes des fractions I et III sont vraisemblablement sous la dépendance d'un même facteur. Les fractions I et III contenant un antigène commun B_2, on est autorisé à penser que cet antigène est un facteur anticoagulant. A l'appui de cette hypothèse, l'expérience démontre que le facteur anticoagulant des fractions I et III et l'antigène B_2 ont la même sensibilité thermique.

On constate, enfin, que la neurotoxine α thermorésistante est représentée par l'antigène D_3. C'est à cet antigène que correspond le polypeptide récemment isolé dans un état voisin de la pureté par Karlsson, Eaker et Porath (10). Le tableau I résume ces observations.

TABLEAU I — ANTIGÈNES DU VENIN DE *NAJA NIGRICOLLIS*

Code	Fonction presumée
a	?
A	Phospholipase
$1B_1$	Ester hydrolase
$3B_1$	?
B_2	Facteur anticoagulant
$1B_3$	?
$3B_3$	Facteur hémolytique direct
$1B_4$	Facteur coagulant
b	?
C	Anti enzyme?
D_1	?
D_2	?
D_3	Toxine α

On peut considérer, pour conclure, que l'antigène A iso-électrique à pH 8,2 et l'antigène fortement basique D_3 représentent les protéines responsables, la première, de l'action hydrolysante du venin sur les lecithines et la seconde de son

action neurotoxique. On est conduit à admettre, d'autre part, qu'un certain nombre d'arguments plaident en faveur des relations de causalité que nous avons tenté d'établir entre divers antigènes et certaines activités enzymatiques exercées. Il apparaît cependant, que les propriétés physiologiques et biochimiques de plusieurs antigènes demeurent indéterminées et que le nombre des antigènes révélés par le phénomène immunologique de la précipitation est inférieur au nombre des activités actuellement connues du venin de *Naja nigricollis*. L'expérience nous enseignera dans quelle mesure une protéine est capable d'exercer plusieurs activités, dans un venin.

La question se pose également de savoir quelles relations peuvent être établies entre les antigènes caractérisés dans le venin de *Naja nigricollis* et les antigènes des autres venins de serpents. Des recherches immunologiques en voie de développement montrent que les venins de deux ELAPIDAE, *Naja haje* et *Naja naja* et celui d'un VIPERIDAE, *Echis carinatus* contiennent des protéines nocives dont la constitution est vraisemblablement voisine de celle de la toxine α. Dans cet ordre de faits, l'étude de la structure de cette "toxine" entreprise à Uppsala par Karlsson, Eaker et Porath, apportera l'élément essentiel d'une solution à ce problème.

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Discussion

C. Y. Lee: "Is there any cardiotoxic component in the venom of *Naja nigricollis*? What is the molecular size of your neurotoxin?"

P. Boquet: "La présence de cardiotoxine n'a pas été recherchée. En ce qui concerne le poids moléculaire de la toxine, nous avons donné seulement le coefficient de sédimentation en unités Svedberg. Le Professeur Porath vous donnera de plus amples informations sur ce point."

S. Minton: "In which fraction of *Naja nigricollis* venom is the antigen shared with the venom of *Echis carinatus*?"

P. Boquet: "Une communanante antigénique a été mise en évidence entre le venin de *Naja nigricollis* et l'échantillon de venin d'*Echis carinatus* qui nous a été adressé d'Ethiopie. Parmi les antigènes communs au venin de *Naja nigricollis* et au venin d'*Echis carinatus* étudiés, il convient de citer un antigène basique correspondant à l'antigène neurotoxique."

F. Kornalík: "How have you tested the procoagulant and anticoagulant properties of the *Naja nigricollis* venom?"

P. Boquet: "L'action du venin de *Naja nigricollis* sur la coagulation du sang a été mesurée selon les techniques usuelles (détermination du temps de prothrombine, etc...) au moyen du plasma humain."

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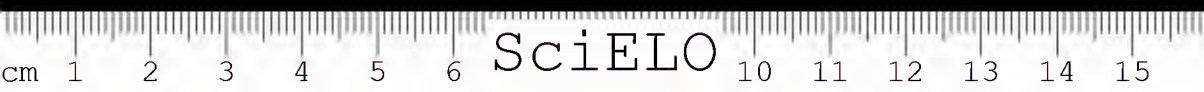
Simp. Internac.
33(2):379-388, 1966

42. SOME SEPARATION METHODS BASED ON MOLECULAR SIZE AND CHARGE AND THEIR APPLICATION TO PURIFICATION OF POLYPEPTIDES AND PROTEINS IN SNAKE VENOMS

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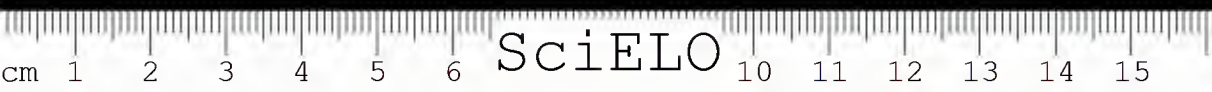
INTRODUCTION

A mathematical problem can often be solved in many ways. One of them might be the most effective, another the most informative, while perhaps all others might appear disgraceful to the mathematicians. An analogous situation often arises when the biochemist is faced with the problem of isolating a particular substance from a complicated mixture such as, for example, an animal venom. Many separation methods are available but a proper choice among them is difficult. Another question concerns the selection of order when several methods are required. I cannot give general answers but only express some personal opinions in the hope to guide those with limited experience in these matters.

It is a commonplace misunderstanding that a method which most effectively resolves a complicated mixture in a single step of necessity also is superior to those of less separation power. Although this is usually true when the purpose is entirely analytical it is not necessarily the case in preparative fractionation. I can mention gel electrophoresis as an example. Analytical electrophoresis in thin layers of starch gel or polyacrylamide has been extremely useful for studies of genetically determined variations in protein patterns of biological fluids and extracts. Nevertheless, in spite of the impressive resolving power of this technique it is usually far more practical to use other methods for large scale separations because of the difficulties involved in the transfer of gel electrophoresis to a preparative scale.

Gel electrophoresis is based on at least three physical factors or parameters, viz., charge, molecular size and shape. Fractionation in two or more separate processes often gives better results and affords more information about the physical or chemical properties of the substance to be purified. Actual application of these methods may be found in references (1, 2). Provided that the gel filtration step is carried out in a column calibrated with respect to the molecular size — retention relationships a rough estimation is possible of the molecular dimensions of the substance under study. Electrophoresis performed in an adsorption-free medium permits conclusions about electrical charge of the migrant species. Since electrophoresis and gel filtration are highly reliable and reproducible methods, both can be employed easily on any scale required in laboratory work.

MOLECULAR SIEVING

Several kinds of gel materials are available that provide separation based on molecular size. Sephadex and Biogel, cross-linked dextran and polyacrylamide, respectively, are the most important commercially available gel substance applicable

to fractionation in aqueous solutions. Both of these gels are available in different degree of cross-linkage, thus providing for molecular weights from a few hundred up to several hundred thousands. For substances of even higher molecular size and for particles agarose gel can be used. For snake venom fractionation Sephadex G100, G75, G50 and G25 have been found to be extremely useful.

An almost complete list of references to the gel filtration method can be obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Electrophoresis

The unexperienced may feel discomfort when faced with the problem of choosing among the very many versions of electrophoretic equipment and techniques now available. Yet electrophoresis is simple in practice although not in theoretical details. This is certainly true even for column electrophoresis the method I prefer myself.

Among the numerous apparatuses for electrophoretic fractionation at my disposal at the Institute of Biochemistry in Uppsala I would select the apparatus of Stellan Hjertén (3) for orienting and analytical purposes and the columns described by myself for preparations on any scale in the range of 25 milligrams (4) to 25 grams (5).

The technique developed by Hjertén is a kind of free zone electrophoresis based on a principle first described by Alexander Kolin (6) and independently somewhat later by Hjertén (7, 8). The fractionation chamber consists of a quartz tube a few millimeter in diameter placed between two electrode chambers and separated from them by membranes. During the run, the tube is rotated continually about its longitudinal axis. Samples can be transferred to or withdrawn from the tube by a capillary tubing. Efficient cooling is achieved by immersion of the tube in a water bath. The progressing separation can be followed by a scanning arrangement which measures absorption at 280 mμ.

The salient feature of the method is the revolving tube. By steady rotation of the tube at a constant speed convection is virtually eliminated. A small sample properly introduced (while tube is revolving) gives a stable starting zone. If no voltage is applied, the zone will broaden only very slowly, chiefly by molecular diffusion. A protein zone remains sharp for hours. When an electric field is applied the various components start to move according to their electrophoretic mobilities. At intervals the separation pattern is checked by scanning. At any time can be removed from the rotating tube and analyzed for biological activity, chemical properties, etc. Samples ranging in size from a few μg to a few mg can thus be analyzed.

Unfortunately this apparatus is not yet commercially produced. In certain applications some of the thin-layer electrophoresis techniques are effective substitutes.

The Hjertén electrophoresis method unfortunately cannot yet be scaled up to allow fractionation of the hundreds of milligrams and more required in most preparation procedures. However, electrophoresis in free solution (i.e. containing no solid support) can be performed on a larger scale than is afforded by the

Hjertén approach. This is accomplished in flowing-film electrophoresis, a continuous separation process (9). Because sample can be introduced continuously, the latter method can provide separations on any desired scale if the components differ widely in mobility. Since this method is mainly useful for group separation and does not have the resolving power required for separating very closely related substances it will not be further discussed here. Instead reference is made to the thesis of Hannig (10).

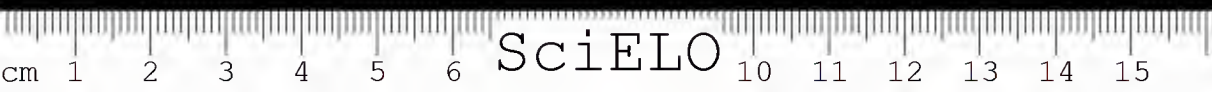
In stationary column electrophoresis the advantage of operating in a uniform, free solution must be sacrificed. A porous powder is introduced into the buffer or a density gradient is produced (11) to suppress macroconvection. Only the first technique will be described here in some detail. It will subsequently be referred to simply as "column electrophoresis".

Column electrophoresis is a discontinuous method. As in the case of revolving tube electrophoresis the sample is introduced in the column before an electric field is applied. The run may be continued for several days at a safe temperature provided that the tube is surrounded with a cooling jacket and the evolution of joule heat is kept low enough to avoid the creation of a large radial temperature gradient. Evidently, column electrophoresis permits the fractionation of substances with similar mobilities.

In my opinion the potentialities of column electrophoresis have been underestimated. I should like to discuss briefly some of the most important drawbacks and supposed inconveniences inherent or adherent to the method.

The first and most important problem is the interference by the support. Adsorption has sometimes been considered to play a substantial role in the fractionation as indicated by the term "electrochromatography". If charged substances adhere to the support by adsorption or precipitation the damage can be disastrous, for this not only increases electroosmosis but also promotes further adsorption. Fortunately, supports with very high degrees of "inertness" are now available, such as specially purified cellulose (from Grycksbo Pappersbruk, Grycksbo, Sweden) and Sephadex.

Another objection to column electrophoresis concerns the difficulty of monitoring the advancement of the migrating zones. Unless they are colored, substances travelling in the non-transparent column cannot be observed during a run. However, substances that have reached the end of the column can be removed continuously by a washing technique and analysed by a recorder before collection (12). This procedure allows monitored withdrawal of the fast-moving substances after which the electrophoresis can be prolonged to separate the components with low mobilities. By proper selection of column length the electrophoresis may be performed very efficiently. Uncharged and charged colored markers may of course be introduced to serve as indices of electroosmosis and to facilitate estimations of the stage of fractionation.

"Column electrophoresis is complicated" is an often heard statement. Those who once have tried the method in our institute never use that argument. It is an extremely simple method whether employed on a moderate (4) or a large scale (5). The LKB column for preparative electrophoresis on a large scale is indeed so simple that I can operate it myself when my technician is ill, although I admit that reading the description in the manual demands intellectual effort (Manual and apparatus are available from LKB Instrument AB, Bromma 1, Sweden).

Ion exchange chromatography

The great break-through for chromatography of proteins came with the introduction of the cellulose ion exchangers by Sober and Peterson (13). Diethylaminoethyl ethers of cellulose (DEAE-cellulose) have proved to be particularly useful. More recently, analogons ion-exchangers have been produced with Sephadex as matrix for example, the DEAE-, CM-(carboxymethyl), and SE-(sulphoethyl) derivatives. The Sephadex exchangers have the advantage over the corresponding celluloses in being more insoluble. This is particularly evident with the cationic types. The large volume changes that accompany alterations in the ionic strength of the medium often complicate chromatography on long ion exchange Sephadex columns.

The popularity that protein chromatography has enjoyed the last decade is due to the easy technical operations and the simple inexpensive equipment required — at least for crude group separations. The more effective elution chromatographic techniques, employing an eluant of constant or continuously changing composition, are more demanding however, because small variations in the experimental conditions often change the elution pattern beyond recognition. Lack of reproducibility is often experienced unless extreme care is taken in the preparation of buffers, in maintaining a constant speed of elution, temperature, etc.

Occasionally, the time and effort expended in searching for optimum conditions for elution chromatography of protein mixtures using an ion exchanger is amply rewarded. Polycarboxylic ion exchangers such as Amberlite IRC-50 have been used with great success to fractionate basic polypeptides and proteins of small molecular size.

Application of gel filtration and ion exchange chromatography to the purification of snake neurotoxins

Isolation procedures

I like to examplify the methods described above with some experiments recently made by Dr. Evert Karlsson and Dr. David Eaker in my laboratory. More detailed descriptions of the procedures will appear later (14). Column electrophoresis has been applied only for orientation purposes, since in this particular case, the isolation of neurotoxins, ion exchange chromatography has a higher resolving power.

But electrophoresis of crude venom gives a good group separation of neurotoxins and phospholipases (Fig. 1).

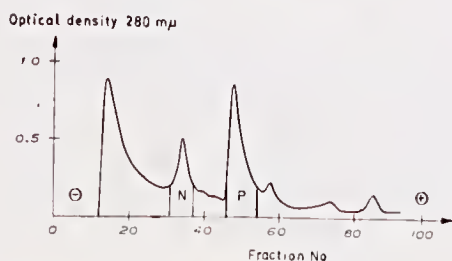



Fig. 1 — Column: Cellulose, Munktell's, 103×3 cm. Buffer: 0.018 M Acetate buffer, 1.50 M Glycine[15] pH 5.0. Sample: 150 mg crude venom. Run: 21 hours, 1200 volt, 35 mA. Field strength 8 volt/cm. Fractions: 5 ml per 30 min. N and P indicate the fractions with the highest neurotoxic and phospholipase activities. (Run made by Dr. Evert Karlsson).

The procedure of isolation of the neurotoxin α of *Naja nigricollis*, the toxin most extensively studied so far, was in short the following:

A sample of 1.1 gram of dry crude *Naja nigricollis* venom dissolved in 10 ml of sodium phosphate buffer of pH 7.3. 0.03 M in sodium ion. This solution was subjected to gel filtration in a Sephadex G75 column (3×80 cm) with elution speed kept at a rate of about 18 ml per hour. 10 ml fractions were collected. Fig. 2 shows a diagram of the same general pattern obtained from an analytical experiment.

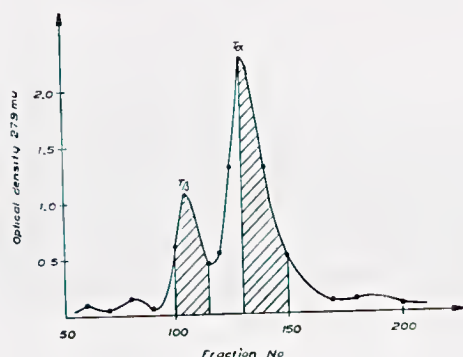



Fig. 2 — Gel filtration of 150 mg crude venom on a 3.2×80 cm column of Sephadex G-75 in 0.03 M sodium-phosphate buffer, pH 7.3. Flow rate: 17.5 ml/hr, 3.5 ml/fraction. $T\alpha$ and $T\beta$ indicate the areas with highest toxic activity.

Pooled fractions containing T_α (see the shaded area in the diagram) were transferred to a 3.2×45 cm column of Amberlite IRC-50 equilibrated with 0.28 M sodium phosphate buffer of pH 7.3. Elution was performed at a rate of 30 ml per hour. Fig. 3 shows the elution pattern obtained in this experiment. About 25 mg of pure neurotoxin T_α was obtained.

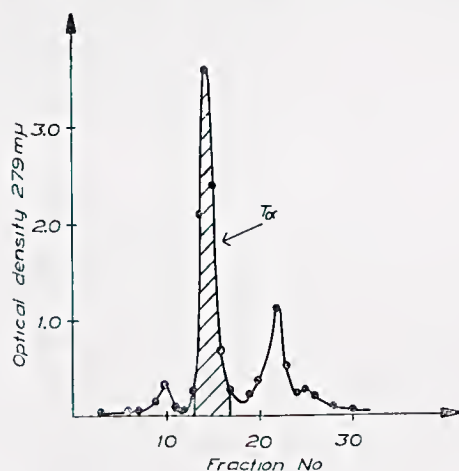



Fig. 3 — IRC-50 chromatography of $T\alpha$ from gel filtration (Fig. 2). Sample applied to 3.2×45 cm column in 250 ml 0.03 M sodium-phosphate buffer. Elution at 30 ml/hr with 0.28 M sodium-phosphate buffer, 6 ml/fraction. Fraction ∅ = 150 ml.

Karlsson and Eaker have adopted this technique for fractionation of other elapidian venoms. Fig. 4 shows the gel filtration diagram of the venom of *Hemachatus haemachatus*. The neurotoxins have about the same elution volume as those of *Naja nigricollis*. When chromatographed on Amberlite IRC-50 with

gradient elution the neurotoxin fraction was resolved into a large number of distinctly different components, many of which were shown to possess neurotoxic activity (Fig. 5).

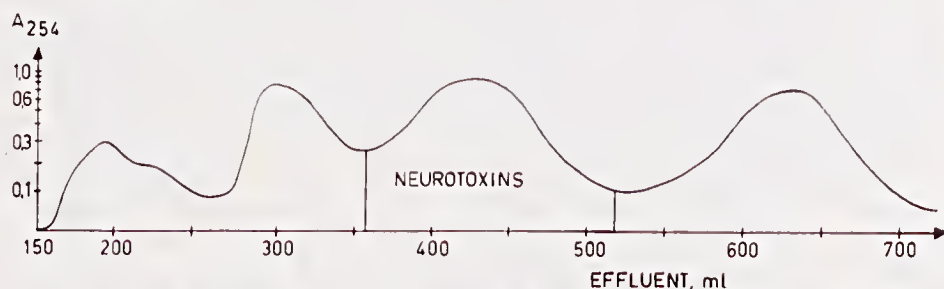



Fig. 4 — Gel filtration of 1.3 g of crude *Hemachatus haemachatus* venom on a 3.2×74.5 cm column of Sephadex G-75 in 0.06 M sodium-phosphate buffer, pH 7.3. Flow rate: 18.8 ml/hour. Effluent monitored continuously at 254 mμ with an LKB Uvicord. The last peak has A_{254} higher than A_{279}.

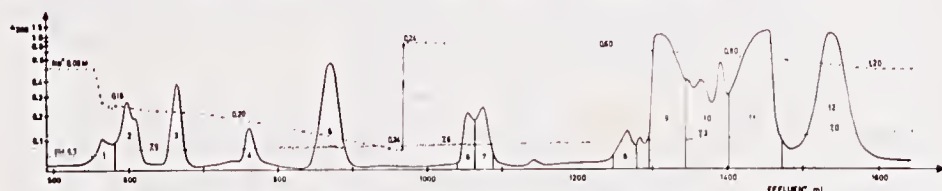



Fig. 5 — Chromatography on Amberlite IRC-50 of fraction "Neurotoxins" (Fig. 4). Column: 3.2×21 cm equilibrated with 0.20 M sodium-phosphate buffer, pH 7.3, and eluted with 50 ml 0.06 molar buffer before application of sample. Run: Performed with a Beckman Model 130 Spectrochrom Analyzer which permits a continuous monitoring of absorbancy, pH and conductivity. After application of sample elution with 0.06 M sodium-buffer was continued until no more unadsorbed material came out. Exponential concave gradient from 0.15 M to 1.50 M sodium. Flow rate 40 ml/hour.

Some properties of the neurotoxins

Neurotoxin T_α of *Naja nigricollis* has a molecular weight of 6787. It consists of 61 amino acid residues lined up in a single peptide chain cross-linked by four disulfide bridges. The neurotoxin T_α is highly basic, as are all the snake neurotoxins studied so far in Uppsala. Thus it moves rapidly to the cathode at pH 8.7 in the Hjertén electrophoresis (Fig. 6). The LD_{100} has been found to be 1.8 μg, as determined in mice of 18-20 g body weight.

In Table I are compiled amino acid analysis data for *Naja nigricollis* T_α, for *Hemachatus haemachatus* components 3, 5 and 12 (Fig. 5), and for the erabutoxins a and b isolated by Tamiya and Arai (16). Peaks 3 and 5 represent highly active neurotoxins (LD_{100} 1.5 and 2.0 μg). The similarity in amino acid composition is remarkable. Further fuel for excited speculation comes from the very similar figures for the *Naja naja atra* toxin of Yang and indeed also for the scorpion toxin described at the Symposium by Dr. Lissitzky. All of these data seem to indicate that a single specific (or a few closely similar) molecular patterns evolved in widely different species is capable of blocking some fundamental physiological process.

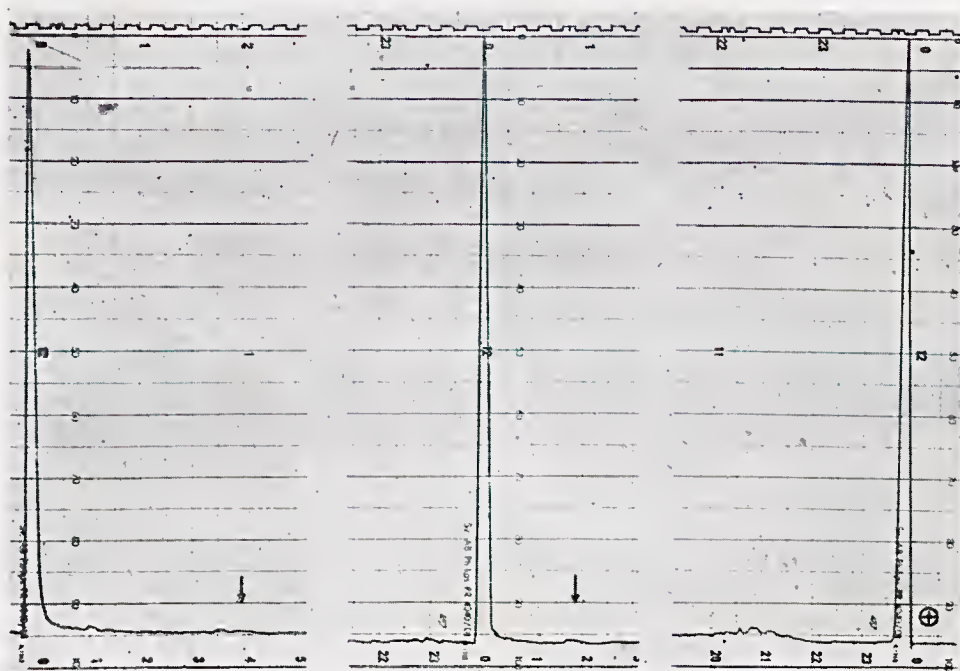



Fig. 6 — Free zone electrophoresis showing electrophoretic homogeneity. Sample: 10 μl of Tα in 0.03 M buffer, pH 7.3, optical density at 279 mμ 1.5. 0.1 M Tris-acetic acid buffer. Run: pH 8.7 (measured at 22°), 3 mA, 1520 V and 1.2°. Exposures: 0.60, and 132 min. after the start. Distance of migration: 17.5 cm. Arrow indicates the starting zone.

TABLE I

Amino acid residues	N.n* Tα	H.h** 3	H.h 5	H.h 12	E.a***	E.b****
Lysine	6	4	6	11	4	4
Histidine	2	2	2	1	1	2
Amide	7	10	8	4	10	10
Arginine	3	5	4	1	3	3
Cysteic acid	—	—	—	—		
Aspartic acid	7	9	5	6	5	4
Methionine Sulfone	—	—	—	—		
Threonine	8	7	9	3	5	5
Serine	2	3	4	3	7	7
Glutamic acid	6	5	8	1	8	8
Proline	5	5	4	5	4	4
Glycine	5	5	5	2	5	5
Alanine	0	0	0	1	0	0
½ Cystine	8	8	8	8	8	8
Valine	2	1	1	4	2	2
Methionine	0	0	0	3	0	0
Isoleucine	3	3	1	2	4	4
Leucine	2	2	2	7	1	1
Tyrosine	1	1	1	1	1	1
Phenylalanine	0	0	0	1	2	2
Tryptophan	1	1	1	0	1 or 2	1 or 2
Total number of amino acid residues	61	61	61	60	61	61

* *Naja nigricollis*
** *Hemachatus haemachatus*
*** Erabutoxin a
**** Erabutoxin b

Min. mol. weight	6787	6828	6823	6707
LD 100, μg (intravenously in mice, 18-20 g)	1.8	1.5	2	50

Acknowledgements — Dr. Paul Boquet directed my interest to the snake venoms. I am most thankful to him and his associates for fruitful collaboration over some years, for the supply of venom and their help in the assays of our fractions with respect to their neurotoxic activity.

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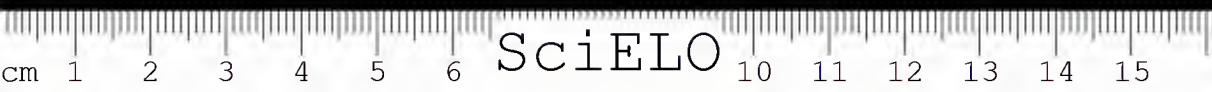
43. SEPARATION METHODS OF ANIMAL VENOMS CONSTITUENTS

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About twenty years ago, the question was raised in our laboratory of whether zinc, which was becoming the object of attention as a metal of biological importance, was present in various snake venoms. At that time, Dr. Delezene reported that many snake venoms contain a considerable amount of zinc. On the other hand, Japanese workers had reported that zinc could not be detected in the venoms of Japanese and Formosan snakes. We re-examined this in thirteen kinds of venom of Japanese and Formosan snakes by emission spectrophotometry, and it was found that all the venoms examined contained considerable amounts of zinc and calcium, a small amount of magnesium, and traces of manganese and other metals. Afterwards, when the effects of cations on the purified enzymes were studied, it was shown that zinc ions markedly inhibit the activities of alkaline phosphatases of the venoms, such as 5'-nucleotidase, phosphomonoesterase, and that calcium ion is an essential component for maintaining the tertial structures of some physiologically active proteinases and toxic proteins in the venoms.

In this way, step by step, our efforts were concentrated on separating the different enzymes in snake venoms, and the problems arising from studies on the pharmacological actions of snake venoms stimulated our interest to elucidate the homeostatic mechanisms which were disturbed by the injection of snake venom enzymes. At present, we are making systematic studies on the purification of enzymes in the venoms of snakes in connection with their physiological actions.

At the beginning of these studies, we applied the venoms to paper electrophoresis (1). We found that ELAPIDAE snake venoms generally contain basic proteins while CROTALIDAE snake venoms contain acidic proteins (Fig. 1).

The purification of the phosphodiesterase in these venoms was our next subject. It was essential to separate phosphodiesterase from 5'-nucleotidase to study the structure of nucleic acid and to elucidate the structure of new nucleotides which were isolated from various biological materials (2, 3, 4, 5), and this separation was also necessary for development of fundamental studies on the production of inosinic acid in Japan. Batch tests were carried out to select suitable adsorbents for chromatographic separation of snake venom phosphodiesterases, with reference to the results of paper electrophoresis. Results of two experiments using 2 mg. of each venom can be seen in Figure 2. The enzyme of the venom of the Formosan Cobra, *Naja naja atra* as we had expected, was readily adsorbed on alumina C-γ-gel, calcium phosphate gel, CM-cellulose or Amberlite CG-50. On the other hand, the enzyme of the venom of the CROTALIDAE snake *Agkistrodon halys blomhoffii* ("Mamushi" in Japanese), was readily adsorbed on calcium phosphate gel, alumina C-γ-gel and DEAE-cellulose (6). Therefore, ELAPIDAE
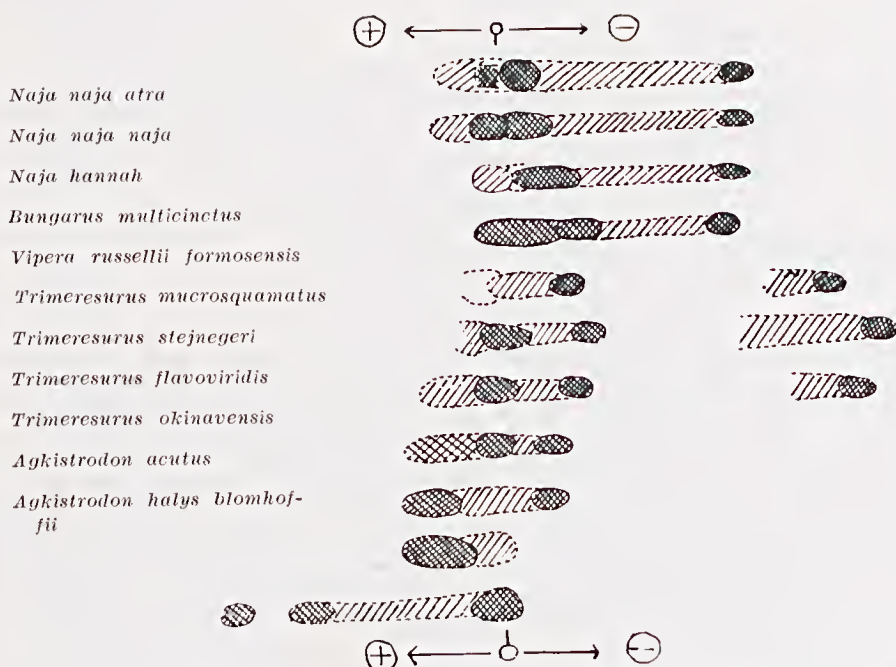
Fig. 1 — Paper electrophoresis of snake venoms. Conditions: Phosphate buffer pH 6.0 μ = 0.1, Toyo Roshi No. 50.
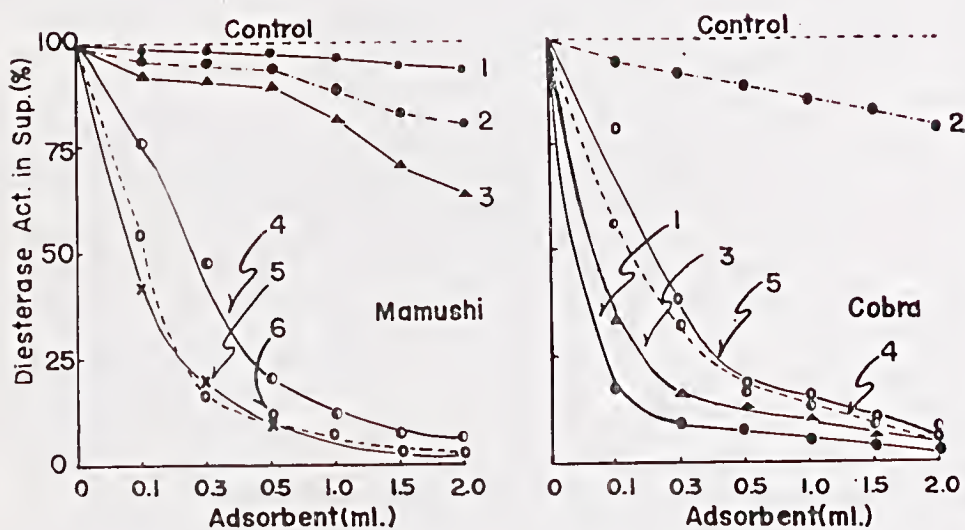
Fig. 2 — Adsorption of Mamushi and Cobra venom Phosphodiesterase on various adsorbents. 1. CM-Cellulose, 2. Cellulose powder, 3. Amberlite CG50, 4. Calcium phosphate gel, 5. Alumina Cγgel, 6. DEAE-Cellulose (Mamushi).

snake venoms were applied to CM-cellulose, alumina C-γ-gel and calcium phosphate gel. Therefore, any of these ion exchangers could be used to obtain phosphodiesterase free from 5'-nucleotidase. On the other hand, "Mamushi" venom was chromatographed on DEAE-cellulose. When the venom was chromatographed on DEAE-cellulose using gradient elution, the phosphodiesterase was separated into three entities. These partially purified enzymes were purified further by chromatography on CM-cellulose, or Sephadex-gel. Thus the three kinds of phosphodiesterases were isolated free from 5'-nucleotidases (7, 8, 9). Table I shows the purified enzymes which were obtained during our studies in the last twelve years.

TABLE I — SNAKE VENOM ENZYMES

Enzymes found in all venoms	
Phospholipase A	L-Amino acid oxidase
Hyaluronidase	Phosphodiesterase
5'-Nucleotidase	Phosphomonoesterase
Endonuclease	Glycerophosphatase
ATPase	
Enzymes found especially in CROTALIDAE *venoms*	
Proteinase	
Enzymes connected with physiological activities of CROTALIDAE *venoms*	
Arginine ester hydrolase	
Enzymes found especially in ELAPIDAE *venoms*	
Acetylcholinesterase Peptidase NADase	

When the venom of *Agkistrodon halys blomhoffii* was applied to a DEAE-cellulose column, phospholipase A was separated into two entities. Similarly other snake venoms contain two kinds of phospholipase A. For example, phospholipase A in Cobra (*Naja naja atra*) venom was also separated into two entities by chromatography on CM-cellulose (10). Moreover, the proteinase and arginine ester hydrolase of "Mamushi" venom were both separated chromatographically into three entities (11,12).

In the course of these studies, it was found that the nucleotide pyrophosphatases, which had been reported to hydrolyze the pyrophosphate linkage of NAD, FAD and coenzyme A, were identical with phosphodiesterases (9), and so, nucleotide pyrophosphatase is not shown in the Table I. The two activities in the "Mamushi" venom were always eluted in the same fraction in chromatographic procedures. So, it is thought that ATPase in this venom corresponds to phosphodiesterase. NADase which decomposes NAD to form nicotinamide and adenosine diphosphate ribose was present in the venom of *Bungarus multicinctus* and *Trimeresurus gramineus*. The NADase in the venom of *Trimeresurus gramineus* was purified by column chromatographies on CM-cellulose and DEAE-cellulose (13).

In this paper, I will report the procedures for purification of proteinases and arginine ester hydrolases, and also some biochemical properties of these purified enzymes. The most characteristic physiological symptoms produced by the injection of the venoms of *A. halys blomhoffii* are connected with proteinases and arginine esterases.

The distribution of proteinases and arginine esterases in Formosan and Japanese snake venoms can be seen in Table II. It was found that proteinase activity is present only in venoms of the CROTALIDAE and that the venoms of ELAPIDAE do not exhibit appreciable proteinase activity. And, it was also found that the hydrolytic activities for benzoylarginine-ethylester (BAEE) are present with the proteinase activity. When the venom was treated with EDTA, the proteinase activity was completely lost while the esterolytic activity remained unchanged. Therefore the enzyme responsible for esterolytic activity must be distinguished from the proteinase activity. And conversely, on treatment with diisopropyl fluorophosphate (DFP), esterolytic activity was completely lost while proteinase activity remained unchanged. The venom of *A. halys blomhoffii*, containing considerable amounts of proteinases and arginine esterases (Table II), was used in further studies.

TABLE II — PROTEINASE AND ARGININE ESTERASE ACTIVITIES IN SNAKE VENOMS

VENOMS		Casein (PU) eas. FR. μg. tyr.	Azocoll	BAEE μ mole/min
CROTALIDAE	*A. halys blomhoffii*	20.2	0.96	13.3
	A. acutus	27.0	3.38	8.8
	T. flavoviridis	19.2	1.14	4.3
	T. mucrosquamatus	26.9	1.4	128
	T. okinavensis	13.2	0.98	11.8
	T. gramineus	9.0	0.14	30
ELAPIDAE	*N. naja atra*	0.12	0,02	0
	N. naja naja	0	0,02	0
	N. hannah	0	0.05	0
	B. multicinctus	0.8	0	0

All the activities were converted to the value for 1 mg. venom.

A typical elution pattern of proteinases of this venom from a DEAE-cellulose column is shown in the Figure 3. Most of the caseinolytic activity was distributed into three fractions and designated as proteinase a, b and c. The recovery of total protein from the column was 98 per cent and approximately 85 per cent of the total caseinolytic activity was recovered in the eluate. The contents of proteinases a, b and c in the crude venom were estimated to be about 0.5, 8.0 and 7.5 per cent, respectively.
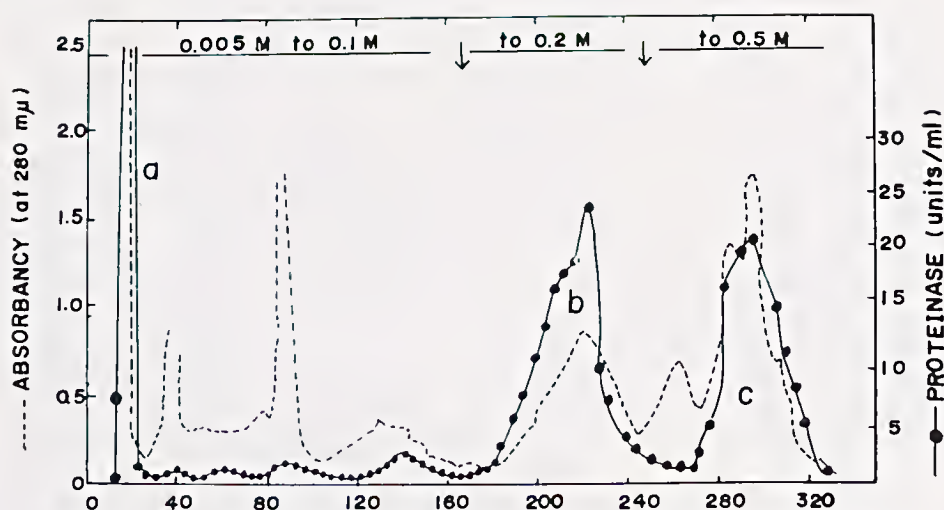
Fig. 3 — Separation of three Proteinases from the Venom of *A. halys blomhoffii* by Chromatography on DEAE-cellulose Column.
Tube Number (10 ml/tube)

Column size	:	2.5×70 cm
Buffer	:	0.005 M ~ 0.1 M ~ 0.2 M ~ 0.5 M CH_3COONa (pH 7.0)
Sample applied	:	3 g of lyophilized venom

Among the three fractions with proteinase activity, only the proteinase b fraction caused hemorrhage when injected intracutaneously in albino rabbits (14). As the hemorrhagic activity of proteinase b could be estimated quantitatively by the method of Kondo and co-workers (15), we attempted to purify proteinase b to verify the correlation of the hemorrhagic and the proteinase activities. After gel-filtration on a Sephadex G-25 column for desalting, the proteinase b fraction was rechromatographed on DEAE-cellulose using gradient elution, and this chromatography gave a single uniform peak of hemorrhagic and proteinase activity (Fig. 4). However, as shown in Figure 4, the curve of absorbancy at 280 mμ did not coincide with the curve of hemorrhagic or proteinase activity. After lyophilization, the sample was applied to a hydroxylapatite column and the hemorrhagic activity was also eluted together with the caseinolytic activity (Fig. 5). To purify the resulting proteinase b preparation, chromatography on DEAE-Sephadex A-25 column was used (Fig. 6). By this procedure, some impurities were removed. The potencies of proteinase b at each purification step are summarized in the Table III. The hemorrhagic and caseinolytic activities of proteinase b were not separated by these purification procedures. The increase in potency of the hemorrhagic activity in each step was essentially in parallel with that of the caseinolytic activity of proteinase b. The purified proteinase b thus obtained was physicochemically homogeneous. Table III shows that the specific activity of the purified preparation is 2.5-fold that of the proteinase b preparation obtained from the first DEAE-cellulose column. The recovery of the proteinase b activity from the crude venom was only 30 to 35 per cent. Therefore, with reference to the chromatographic patterns in Table III, we attempted to simplify the purification method to increase the yield. The proteinase b fraction eluted from the first DEAE-cellulose column was applied to a Sephadex G-100 column (Fig. 7). By
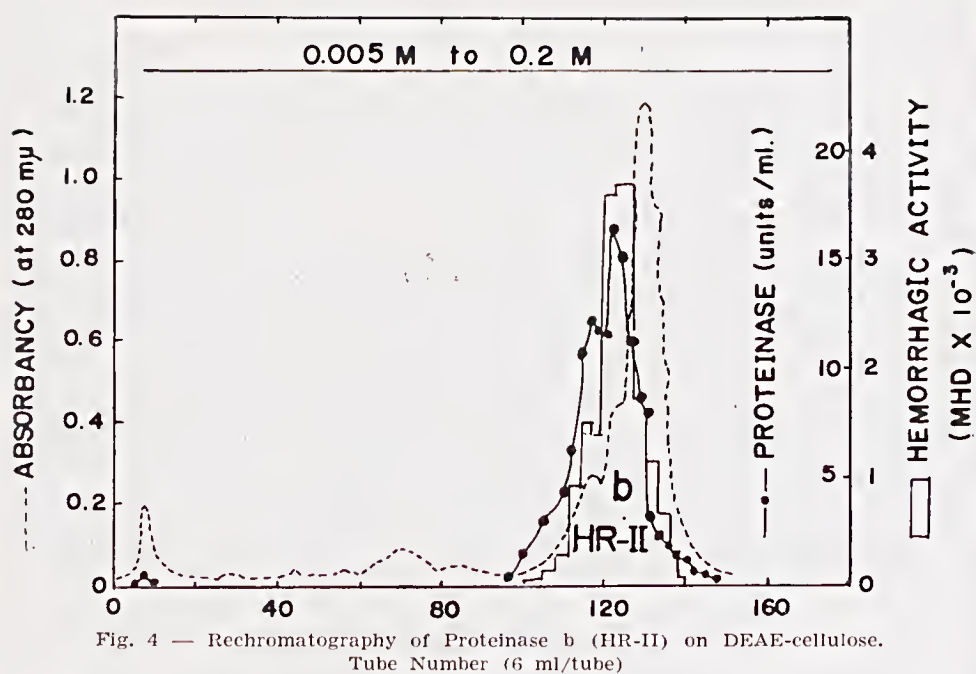
Fig. 4 — Rechromatography of Proteinase b (HR-II) on DEAE-cellulose. Tube Number (6 ml/tube)

Column size	:	1.5×25 cm
Buffer	:	0.005 M ~ 0.2 M CH_3COONa (pH 7.)
Sample applied	:	HR-II fraction separated on DEAE-cellulose (total absorbancy at 280 mμ = 127)
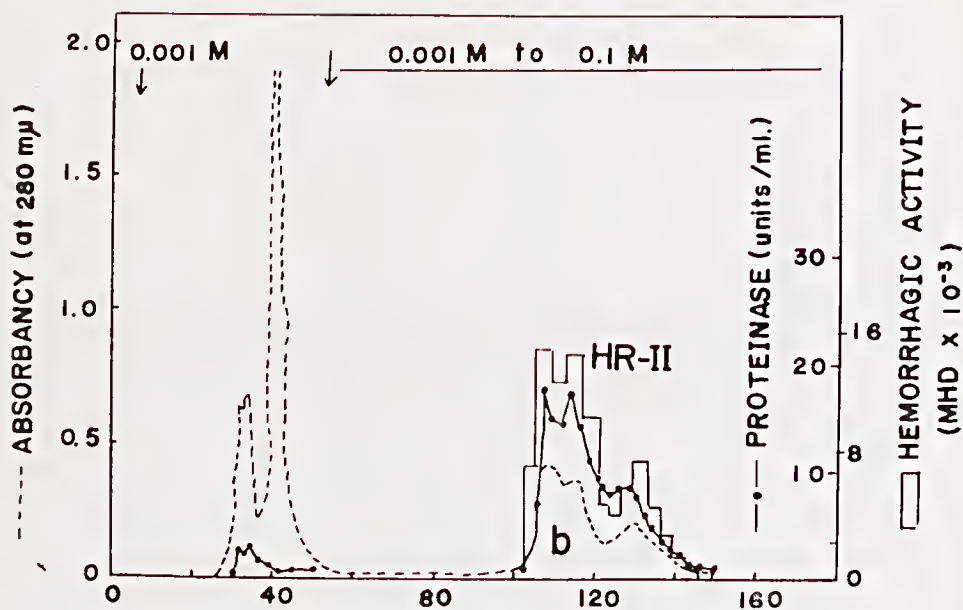
Fig. 5 — Purification of Proteinase b (HR-II) on Hydroxylapatite Column. Tube Number (9.3 ml/tube)

Column size	:	2.5×50 cm
Buffer	:	0.001 M ~ 0.1 M phosphate (pH 7.5)
Sample applied	:	HR-II fraction eluted from DEAE-cellulose (total absorbancy at 280 mμ = 253)
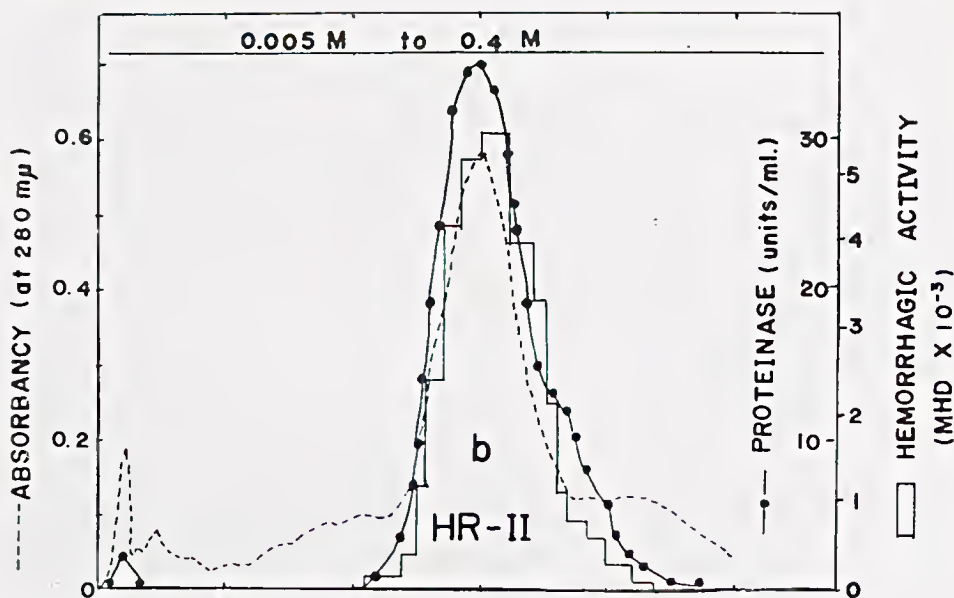
Fig. 6 — Purification of Proteinase b on DEAE-Sephadex A-25 Column.
Tube Number (3 ml/tube)

Column size : 1.5×20 cm
Buffer : 0.005 M～0.4 M CH_3COONa (pH 7.0)
Sample applied : HR-II purified on hydroxylapatite (total absorbancy at 280 mμ=127)
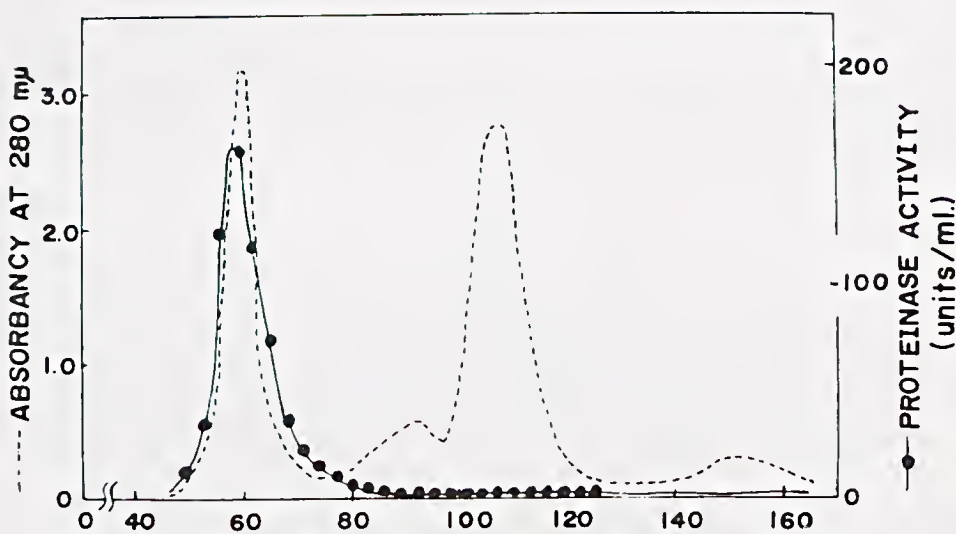
Fig. 7 — Gel Filtration of Proteinase b Fraction on Sephadex G-100.

Tube Number (5.0 ml/tube)
Ten milliliter of 3.48% proteinase b fraction was applied to a column of Sephadex G-100 (3.2×88 cm), equilibrated with 0.05 M AcONa (pH 7.0), and eluted with same buffer. Flow rate was 60 ml per hour at 4°C.

TABLE III — HEMORRHAGIC AND ENZYME ACTIVITIES DURING PURIFICATION OF PROTEINASE b

FRACTION FROM	Hemorrhagic activity		Proteinase activity
	MHD* and its fiducial limits (μg)	Specific activity	(PU) {cas. FR / μg. tyr.} /mg protein
First DEAE-cellulose column	0.48 (0.42-0.57)	2.10	20.2
Second DEAE-cellulose column	0.42 (0.39-0.49)	2.38	33.0
Hydroxylapatite column	0.26 (0.22-0.30)	3.84	40.6
DEAE-Sephadex column	0.19 (0.17-0.23)	5.25	70.5

*MHD: Minimum Hemorrhage Dose when injected in the skin of the back of albino rabbit.

this procedure, proteinase b was eluted in a symmetrical peak. When the proteinase b fraction separated from the column was applied to DEAE-Sephadex A-50, the absorbancy curve at 280 mμ completely coincided with the proteinase activity (Fig. 8). Thus, a simple purification method was achieved increasing the yield of the purified proteinase b (Table IV) and the recovery of proteinase b activity from the crude venom was about 90 per cent. Table V shows the analytical data of the purified proteinase b. Calcium ion is the component of this metal-protein.
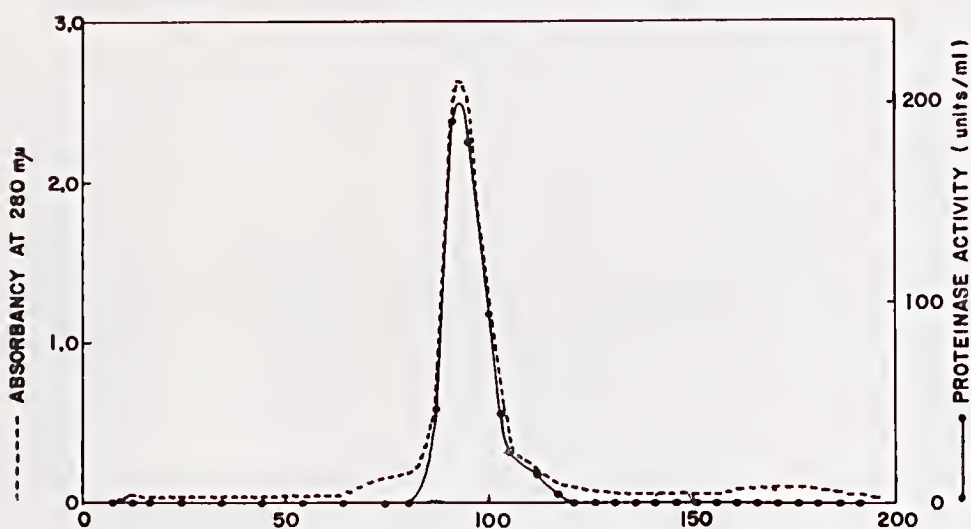
Fig. 8 — Concave Gradient Chromatography of the purified Proteinase b (HR-II) from Sephadex G-100 on DEAE-Sephadex A-50.
Tube Number (3 ml/tube)

Column size : 1.5×29 cm
Mixing chamber : 250 ml of 0.005 M CH_3COONA (pH 7.0)
Reservoir : 0.4 M CH_3COONa
Sample applied : 112.5 mg

TABLE IV — SUMMARY OF PURIFICATION PROCEDURES OF PROTEINASE b

FRACTION	Protein (g.)	Total units	Specific activity
Crude venom	38.4		
DEAE — cellulose eluate	6.238	212,700	34.1
Sephadex G-100 eluate	2.856	197,500	69.2

TABLE V — AMINO ACID COMPOSITION AND CARBOHYDRATE CONTENT OF PROTEINASE b (HR-II)

Per cent of amino acid residue				Per cent of carbohydrate residue	
Gly	2.58	Tyr	3.86	Galactose	6.0
Ala	2.82	Try	1.83		
Ser	2.74	Cys/2	4.54	Mannose	2.0
Thr	3.95	Met	8.00		
Pro	3.25	Asp	11.79	Fucose	trace
Val	4.10	Glu	8.43		
Ileu	4.55	Amido N	2.06	Glucosamine	6.5
Leu	5.44	Arg	4.12	Sialic acid	3.0
Phe	2.67	His	2.37		
		Lys	3.99	Calcium	0.32
Total A.A. residue			77.69	Total sugar residue and calcium	17.82

The hemorrhagic and caseinolytic activities of proteinase b was inhibited by EDTA (Table VI), and the decrease in biological activity was parallel with the amount of EDTA added. The proteinase activity decreased as the calcium ions were removed by electrodialysis (Fig. 9). and consequently a conformational change of proteinase b was observed by measurement of the difference spectrum of the protein. The activity was not regained even when calcium ion was added to this dialyzed deionized protein.

TABLE VI — EFFECT OF EDTA ON PROTEINASE b (HR-II)

EDTA (Final M)	HEMORRHAGIC ACTIVITY		Proteolytic activity
	MHD/μg. protein	Relative value (%)	Relative value (%)
None	3.6	100	100
1×10^{-4}	2.4	67	29
5×10^{-4}	0.8	22	6
1×10^{-3}	0.5	14	2
5×10^{-3}	0.1	3	0
1×10^{-2}	0	0	0

130 μg of proteinase b were treated with the indicated amounts of EDTA for 30 minutes at 37°C in a total volume of 1.0 ml.
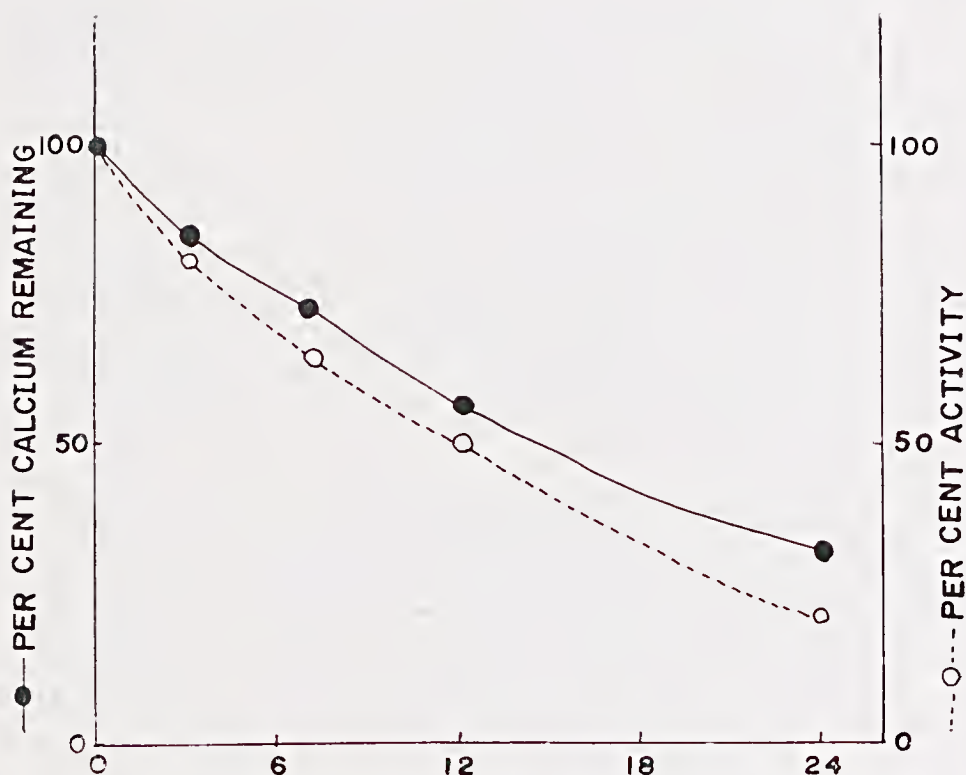
Fig. 9 — Dependence of Proteinase b Activity on Calcium.

Electrodialysis Time (hours)
Protein concn: 0.44%, Voltage: 400 V, Current 1.9 ~ 0.9 mA
Flow rate of deionized water was 25 ml/hr at 4°C

We also attempted to purify proteinase c. The content of proteinase c in the crude venom of *A. halys blomhoffii* was about 7.5 per cent. Similar procedures to those used in the purification of proteinase b were applied. The results can be seen in Table VII. The preparation obtained from a Sephadex

TABLE VII — PURIFICATION METHOD OF PROTEINASE c

FRACTION	Protein (g.)	Total units *	Specific activity **
Crude venom	36.0		
DEAE-cellulose eluate	6.93	163,548	23.6
Sephadex G-100 eluate	2.346	174,308	74.3

* One unit was defined as the amount of enzyme which yields a color equivalent to 1.0 μg of tyrosine per minute using casein as substrate.

** Specific activity is expressed as units per mg protein.

G-100 column was electrophoretically and ultracentrifugally homogeneous and so proteinase c was also purified by this simple method. When 10 μg of proteinase c were injected subcutaneously into the skin of a depilated albino rabbit, a marked edema was observed. Injection of equal amounts of bromelain, papain, plasmin, α-chymotrypsin or bacterial proteinase obtained from *Bacillus subtilis*, did not cause edema. It is not clear by what mechanism proteinase c causes this edema.

Only 0.5 per cent of the crude venom of *A. halys blomhoffii* corresponds to the proteinase a.

Phospholipase A, L-amino acid oxidase, hyaluronidase and various other proteins were found as contaminants of protease a in the eluate of the first DEAE-cellulose column (Fig. 3). To remove these contaminants, this fraction was submitted to a purification procedure (Table VIII). The recovery of proteinase a

TABLE VIII — PURIFICATION PROCEDURES OF PROTEINASE a FROM THE VENOM OF *A. HALYS BLOMHOFFII*

1. DEAE-Cellulose Chromatography of Crude Venom, Proteinase a Fraction
↓
2. Phospho-Cellulose Chromatography
↓
3. Sephadex G-25 Gel Filtration
↓
4. Second DEAE (pH 8.5) Chromatography
↓
5. Third DEAE (pH 7.0) Chromatography
↓
6. Sephadex G-100 Gel Filtration
↓
Purified Proteinase a

activity was about 70 per cent of that in the crude venom. The physiological activity of proteinase a is not yet known. The electrophoretic and ultracentrifugal patterns showed that each purified proteinase appears as a single protein (Fig. 10 and 11). In addition to these criteria of purity, the homogeneity of each proteinase was also given by the overlapping curves of absorbancy at 280 mμ and proteinase activity (Fig. 12). Moreover, these purified proteinases were homogeneous on polyacrylamide gel electrophoresis.

The optimum pH values of each proteinase can be seen in Figure 13.

The substrate specificities of purified proteinases a, b and c were examined using synthetic glucagon and B chain of oxidized insulin from bovine origin (Fig. 14). These proteinases are considered as digestive enzymes of the snake, however, they differ from the usual mammalian digestive enzymes, trypsin and chymotrypsin in their hydrolytic actions. Although the venom proteinases have specificities similar to those of bacterial proteinases, their hydrolytic sites are restricted within narrow limits. Proteinase b is the bradykinin destroying enzyme in the venom, and it readily hydrolyzes the glycylphenylalanyl linkage of bradykinin and of the B chain of insulin. Although proteinase c hydrolyzes the glycylphenylalanyl linkage of the B chain easily, it did not hydrolyze the glycylphenylalanyl linkage of bradykinin.
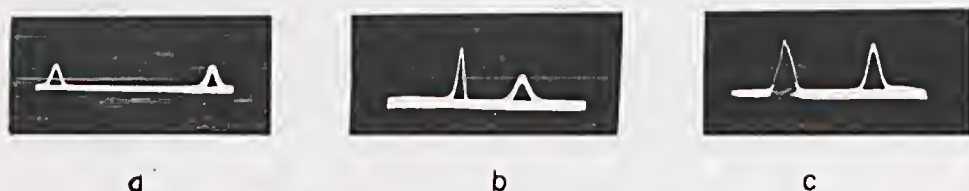
Fig. 10 — Electrophoretic Patterns of Purified Proteinase a, b and c.

Electrophoresis was carried out at conditions described below; proteinase a: 1.0%, pH 8.60, μ = 0.1, 6.14 mA, 3.3°C; proteinase b: 0.92%, pH 8.51, μ = 0.1, 6.10 mA, 4.6°C; proteinase c: 0.80%, pH 6.00, μ = 0.1, 6.00 mA, 6.0°C. The pictures were taken after 150, 120 and 125 minutes on proteinase a, b and c, respectively.
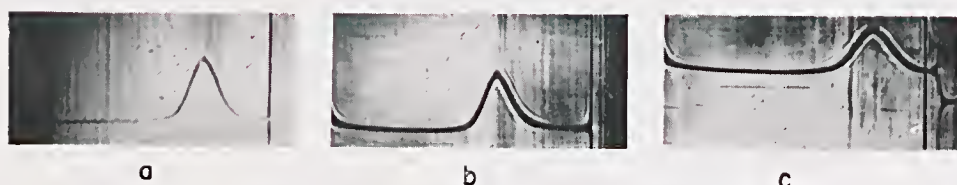
Fig. 11 — Ultracentrifugal patterns of purified proteinase a, b, and c.

A solution of sample in sodium phosphate buffer (pH 7.0, μ = 0.1) was run at conditions described below; proteinase a: 0.75%, 55,430 rpm, 25.7°C; proteinase b: 0.65%, 56,100 rpm, 16.6°C; proteinase c: 0.77%, 56,100 rpm, 16.6°C. The pictures were taken after 48, 64 and 64 minutes on proteinase a, b and c, respectively.
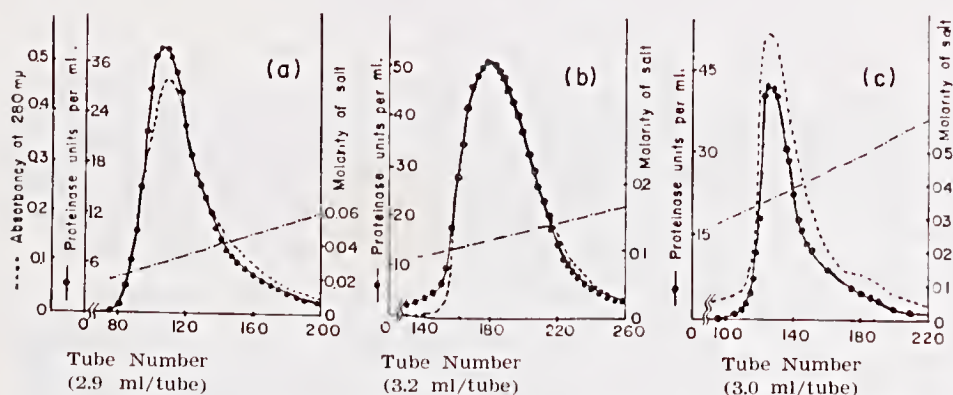
Fig. 12 — Linear Gradient Elution of Purified Proteinase a, b and c by DEAE-cellulose Chromatography.

Column size	:	1.5×24 cm
Buffer	:	a 0.001 M Tris (pH 8.5) to 0.06 M CH_3COONa (pH 7.0)
		b 0.01 M CH_3COONa (pH 6.2) to 0.2 M CH_3COONa (pH 6.2)
		c 0.05 M CH_3COONa (pH 6.2) to 0.5 M CH_3COONa (pH 6.2)
Sample applied	:	a = 80.4 mg, b = 99.0 mg, c = 69.7 mg

Fig. 13 — Optimum pH of Venom proteinases.

Fig. 14 — Amino Acid Sequence of Oxidized Bovine Insulin B-chain and the Sites of Hydrolysis by Snake Venom Proteinases.
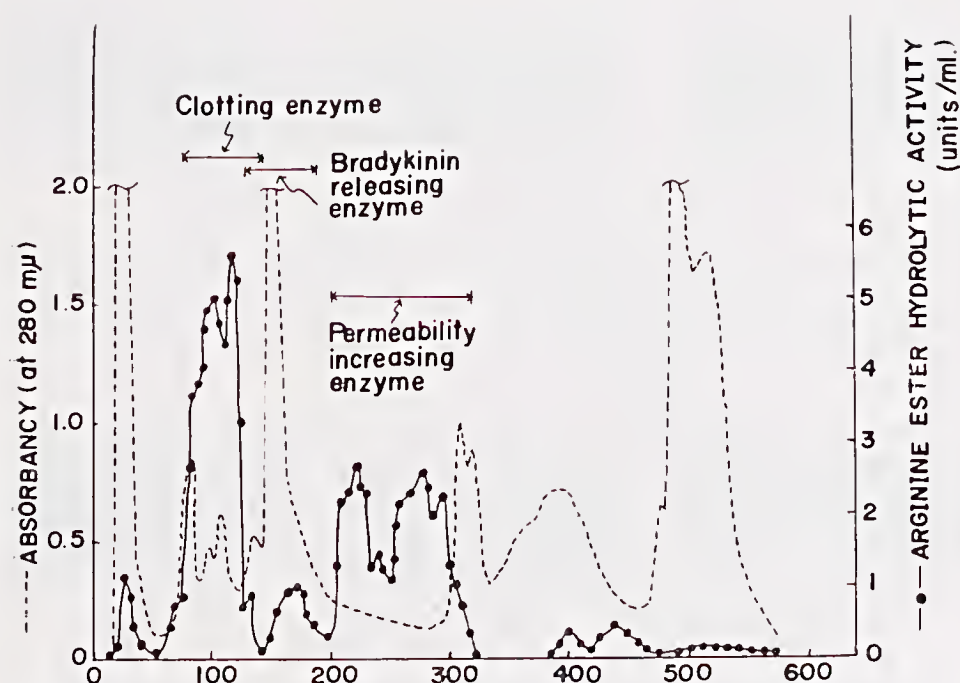
Fig. 15 — Chromatography of the Venom of *A. halys blomhoffii* on DEAE-cellulose.

Tube Number (20 ml/tube)
Column size: 5.0×35 cm. Buffer, CH_3COONa pH 7.0. Venom applied, 6 g.

We also attempted to purify the "clotting enzyme" and the "capillary-permeability-increasing enzyme" which were present in considerable amounts in the eluate of the DEAE-cellulose column (Fig. 15). The blood clotting enzyme fraction free from the hemorrhagic proteins and other small impurities was obtained after rechromatography on a DEAE-cellulose column (Table IX) followed by fractionation on a hydroxylapatite column (Fig. 16). After concentration and desalting by Sephadex G-25 filtration, the blood clotting enzyme fraction was applied to a column of DEAE-Sephadex A-25, and eluted by concave gradient elution (Fig. 17). By this procedure the clotting enzyme was purified to a

TABLE IX — SUMMARY OF PURIFICATION PROCEDURES USED FOR THE "CLOTTING ENZYME"

FRACTION FROM	Total protein A. at 280 mμ	Enzyme units *	Specific activity
First DEAE-cellulose column	1,242	1,849	1.5
Second DEAE-cellulose column	106	1,541	14.5
Hydroxylapatite column	42	1,392	33.2
DEAE-Sephadex column	22.3	788	35.3

* Tosylarginine methylester was used as substrate.
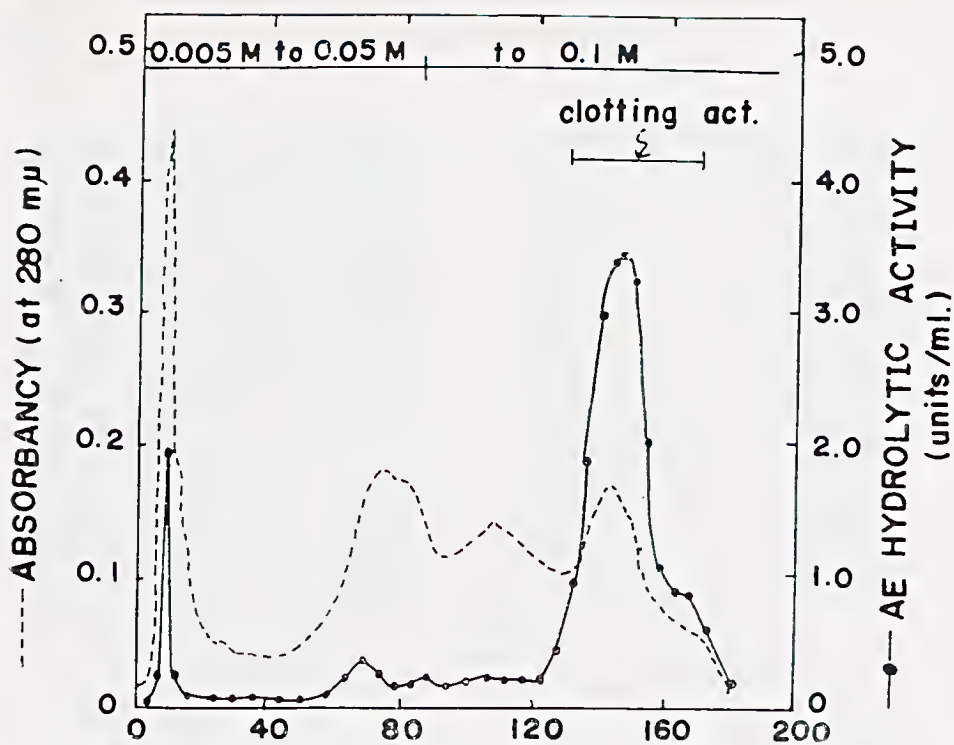
Fig. 16 — Fractionation of the "Clotting Enzyme" on Hydroxylapatite Column.

Tube Number (5.5 ml/tube)

Column size : 1.5×20 cm
Buffer : 0.005 M ~ 0.05 M ~ 0.1 M phosphate (pH 6.8)
Sample applied : Clotting enzyme fraction (total absorbancy at 280 mμ = 108)
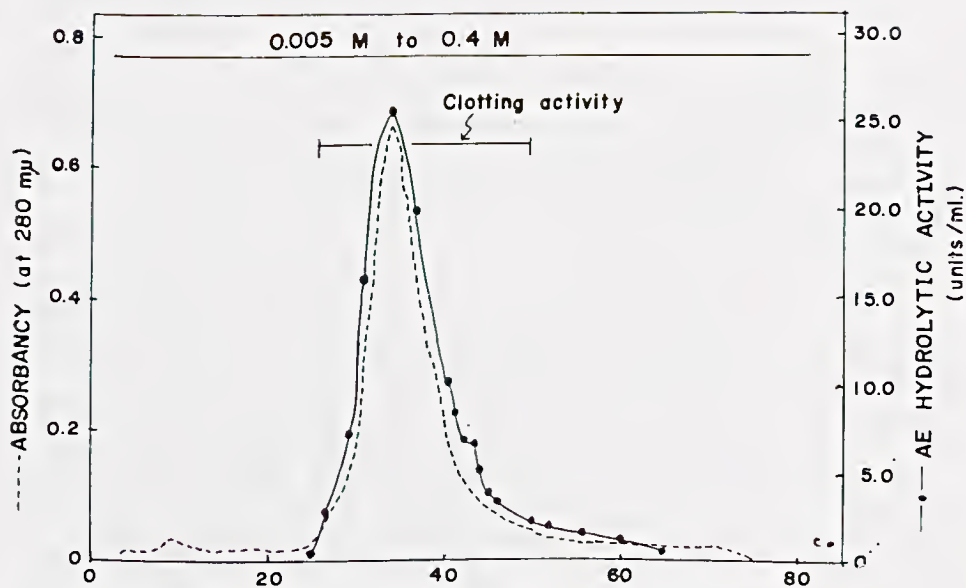
Fig. 17 — Elution Pattern of Purified "Clotting Enzyme" on DEAE-Sephadex A-25.

Tube Number (5 ml/tube)

Column size : 1.8×30 cm
Sample applied : 0.005 M ~ 0.4 M CH_3COONa (pH 7.0)
Buffer : Purified clotting enzyme (total absorbancy at 280 mμ = 42)

physicochemically homogeneous state and Fig. 18 shows the ultracentrifugal pattern.

As mentioned before, among the arginine ester hydrolases in the eluate from the first DEAE-cellulose column (Fig. 3), an enzyme was found which has a hypotensive action and increases capillary permeability. The arginine ester hydrolytic activity of this enzyme amounted 30 to 40 per cent of the total arginine ester hydrolytic activity of the venom. This physiological active arginine hydrolase was purified by a similar method to that used for the clotting enzyme. The results are summarized in Table X. The specific activity of this enzyme increased

TABLE X — SUMMARY OF PURIFICATION PROCEDURES USED FOR THE "CAPILLARY PERMEABILITY INCREASING ENZYME"

FRACTION FROM	Total protein A. at 280 mμ	Enzyme units *	Specific activity
First DEAE-cellulose column	1,030	2,440	2.37
Second DEAE-cellulose column	314	1,916	6.1
Hydroxylapatite column	65.8	1,363	20.7
DEAE-Sephadex column	29.0	837	28.9

* Tosylarginine methylester was used as substrate.

markedly and the purified preparation was homogeneous on ultracentrifugation (Fig. 19), and also on cyanogum electrophoresis at various pH values. When 3 μg of this purified preparation were injected into the skin of an albino rabbit, capillary permeability was distinctly increased, as judged by the Evans Blue test. When this preparation was incubated with purified bradykininogen, no release of bradykinin was detected by assay on guinea-pig ileum, and so it is not clear by what mechanism the permeability of the capillaries is increased. In other experiments, we found that a considerable amount of this capillary permeability increasing enzyme was also present in *Crotalus adamanteus* venom and *Trimeresurus flavoviridis* venom.

The fractions in the eluate from the first DEAE-cellulose column (Fig 3) which contained "bradykinin releasing", and "clotting" activities were collected, and rechromatographed. Then the fraction of the eluate with "bradykinin releasing activity" was purified further on a CM-cellulose column (Fig. 20). In this way the "bradykinin releasing enzyme" was obtained free from clotting enzyme. but only 5 per cent of the total units of arginine ester hydrolytic activity of the venom were recovered in this partially purified enzyme preparation, and no further purification was attempted. Although, the arginine ester hydrolytic activity of this purified enzyme was unexpectedly low, the activity in this preparation seems to be linked to the bradykinin releasing activity (16). On heat-treatment, the bradykinin releasing activity decreased parallel with the arginine ester hydrolytic activity. Also, in DFP-inhibition experiments, the bradykinin releasing activity decreased parallel with the arginine ester hydrolytic activity, with increase in the concentration of DFP.

Fig. 18 — Ultracentrifugal Pattern of the "Clotting Enzyme".

A solution of the purified "clotting enzyme" at a concentration of 1.0 per cent in sodium phosphate buffer, pH 7.5, ionic strength, 0.1 μ was run at 58,100 rpm at 19.4°C and the picture was taken after 52 minutes centrifugation.

Fig. 19 — Ultracentrifugal Pattern of the "Capillary Permeability Increasing Enzyme".

A solution of the purified "capillary permeability increasing enzyme" at a concentration of 1.0 per cent in sodium phosphate buffer, pH 7.5, ionic strength, 0.1 μ was run at 57,800 rpm at 21.2°C, and the picture was taken after 55 minutes centrifugation.
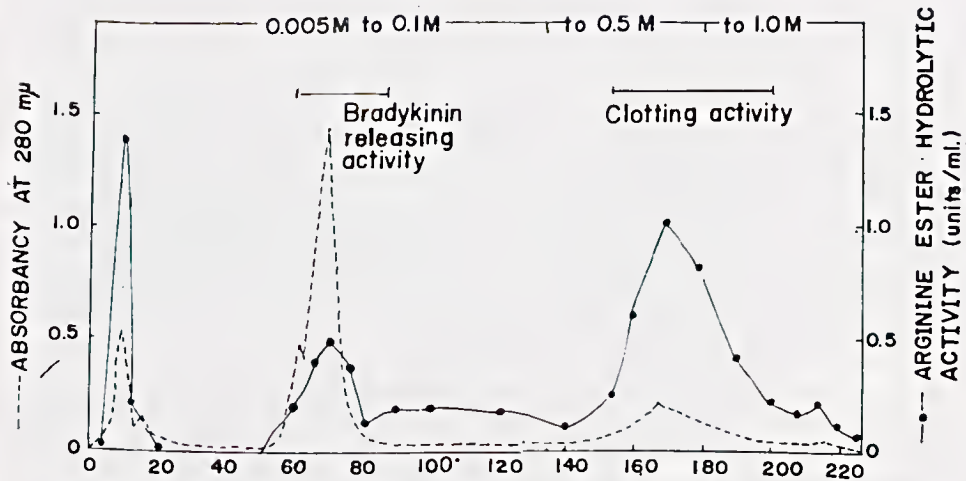
Fig. 20 — Purification of Bradykinin Releasing Enzyme on CM-Cellulose Column.

Tube Number (5 ml/tube)

Column : 1.5×32.5 cm
Buffer : CH_3COONa, pH 6.0
Protein applied : total absorbancy at 280 mμ = 134

The effects of synthetic substrates can be seen in Table XI. The inhibition caused by benzoylarginine ethylester depended on its concentration, and the bradykinin releasing activity was completely inhibited when a sufficient amount of benzoylarginine ethylester was added. Lysine ethylester and benzoylargininamide, which were not hydrolyzed by the bradykinin releasing enzyme of the venom, did not inhibit its activity.

TABLE XI — EFFECT OF SYNTHETIC SUBSTRATE ON THE PURIFIED BRADYKININ RELEASING ENZYME

Synthetic substrate added	Final concentration of substrate (M)	Per cent of bradykinin releasing activity
	None	100*
BAEE	2.5×10^{-7}	100
	2.5×10^{-6}	68
	2.5×10^{-5}	40
	2.5×10^{-4}	10
	2.5×10^{-3}	0
LysEE	2.5×10^{-3}	100
BAA	2.5×10^{-3}	100

* When inhibitor was not added, the amount of bradykinin was estimated to be 0.8 μg upon guinea-pig ileum. 25 μg of the enzyme was used.

Trasylol, which is a potent inhibitor of urinary and pancreatic kallikreins, caused distinct inhibition of the activity of the bradykinin releasing enzyme of the venom. However, it inhibited the arginine ester hydrolytic activity of the crude venom only slightly. We thought that trasylol would probably not inhibit the activities of the "clotting" and "capillary permeability increasing" enzymes of the venom, and this was next examined and found to be the case. Thus, the inhibitory action of trasylol is specific for the bradykinin releasing enzyme. From these results and the specificities of the enzymes for various synthetic substrates, it seems that the bradykinin releasing enzyme in the venom is a salivary kallikrein of the snake.

I would also like to mention two tri-pyroglutamyl peptides which were recently isolated from the venoms of CROTALIDAE (17).

When the venom of *A. halys blomhoffii* was applied to a DEAE-cellulose column and eluted with 0.005 M to 0.1 M acetate buffer at pH 7.0 a third main peak with absorption at 280 mμ was eluted. This fraction contained peptides of low molecular weight and its absorbancy at 280 mμ was about 10 per cent of that of the unfractionated venom. The UV-spectrum of the peptide fraction was similar to that of tryptophan. Table XII, shows the procedure used to purify the peptides. After removing protein contaminants by gel-filtration on a column of Sephadex G-25, the eluted peptide fraction was applied to a column of DEAE-

TABLE XII — PURIFICATION PROCEDURES OF TRYPTOPHAN-CONTAINING PEPTIDES FROM THE VENOM OF *A. HALYS BLOMHOFFII*

	Step	Total E_{280}	Dry Weight
1	Crude Venom	40875	32.72 g
2	DEAE-cellulose Column	5040	
3	Sephadex G-25	3090	
4	DEAE-Sephadex A-25	2200	
5	DEAE-cellulose Column	2010	
6	DEAE-Sephadex A-25	1696	160.00 mg

Sephadex A-25, and absorbed materials were eluted by 0.5 M pyridine acetate buffer at pH 5.0. Final purification was achieved by rechromatographies of the partially purified peptide fraction on DEAE-cellulose and DEAE-Sephadex A-25.

At the beginning of this work, as only a single spot was seen on paper or thin layer chromatography or paper electrophoresis of this peptide fraction, which stained with Ehrlich's reagent, we thought there was only one peptide. Acid hydrolysis of the sample in constant boiling HCl in an evacuated sealed tube, yielded aspartic and glutamic acids, ammonia and traces of tryptophan. Sanger's technique failed to reveal a free N-terminal residue. and C-terminal analysis by hydrazinolysis gave only tryptophan. Pyroglutamic acid was identified in partial hydrolyzates prepared with N-NaOH or 0.1 N-HCl. After digestion with carboxypeptidase A, tryptophan and two fragments. pyroglutamylasparagine and pyroglutamylglutamine, were separated from the reaction mixture, and these dipeptides were separated from each other by high voltage electrophoresis (Fig. 21). They were identified by comparison with synthetic samples.

1. Synthetic Pyroglu-Asp(NH_2) + Try
2. CPase Digest of the Sample
3. Synthetic Pyroglu-Glu(NH_2) + Try

Fig. 21 — Comparison with CPase Digest of Tryptophan-containing Peptides and Synthetic Samples.

High voltage electrophoresis was carried out at pH 3.5 at 3000 V per 60 cm for 120 min.

The above results suggest that there are two peptides of similar electrophoretic mobility in the peptide fraction. These two components were separated from the peptide fraction by high voltage electrophoresis (Fig. 22). Using ninhydrin and
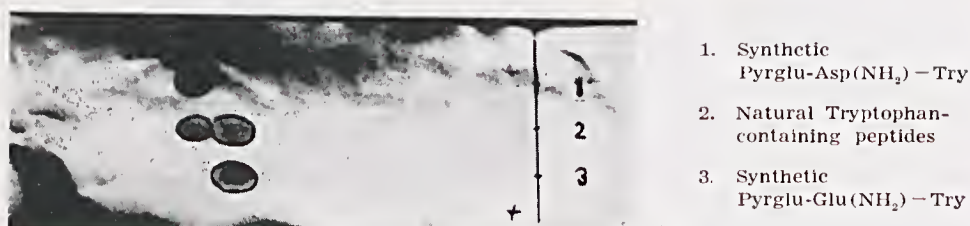
Fig. 22 — Comparison with Synthetic and Natural Peptides.
High voltage electrophoresis was carried out at pH 3.5 at 4000 V per 60 cm for 240 min.

microbiological methods, the molar ratio of amino acids in the acid hydrolyzate of one of these two peptides was established as L-Tryptophan:L-Glutamic acid = 1:2 and that of the other as L-Tryptophan:L-Glutamic acid:L-Aspartic = 1:1:1. Thus, the sequences of the two peptides were deduced to be Pyro-Glu (NH2)-Try and Pyro-Asp (NH2)-Try, respectively.

Next, the distribution of these peptides in various snake venoms was examined (Table XIII). The venoms of *Crotalus adamanteus, Bothrops jararaca* and *Trimeresurus flavoviridis* contained both peptides, while the venom of *Vipera russellii* contained only one of them, and in the venom of *Naja naja atra* neither of the two peptides was found. The significance of these peptides in the salivary gland of poisonous snakes is obscure. It is tempting to speculate that these peptides are originated from precursors of enzymes or biologically active peptides, which are present in especially high concentrations in CROTALIDAE and VIPERIDAE venoms, during activating processes.

TABLE XIII — DISTRIBUTION OF TRYPTOPHAN-CONTAINING PEPTIDES IN VARIOUS SNAKE VENOMS

SNAKE		Pyrglu-Glu(NH_2)-Try	Pyrglu-Asp(NH_2)-Try
A. halys blomhoffii	(CROTALIDAE)	+	+
A. piscivorus piscivorus	(CROTALIDAE)	+	+
T. flavoviridis	(CROTALIDAE)	+	+
C. adamanteus	(CROTALIDAE)	+	+
B. jararaca	(CROTALIDAE)	+	+
V. russellii	(VIPERIDAE)	—	+
N. naja naja	(ELAPIDAE)	—	—

The work I have reported in this paper is mainly connected with the pharmacological and enzymatic activities of Japanese snake venoms. I feel that pharmacological investigations on snake venoms are complicated by the fact that these venoms are what you might call solutions of toxins in saliva, and snake venom

contains many components with physiological activities. Therefore, I believe that for the elucidation of the physiological actions of snake venoms on a molecular basis, it is important to purify each component from each snake venom.

We now have at hand several techniques for this purpose. Chromatography on ion-exchangers and gel-filtration are excellent methods for the purification of pharmacologically active proteins. All these methods have their limitations, but fortunatelly the limitations are not the same for the varions materials. By combining appropriate chromatographical and gel-filtration procedures and other techniques, it may be possible to purify many active components. We hope that further research will be planned in future to give much clearer results than those which were possible before this International Symposium on Animal Venoms.

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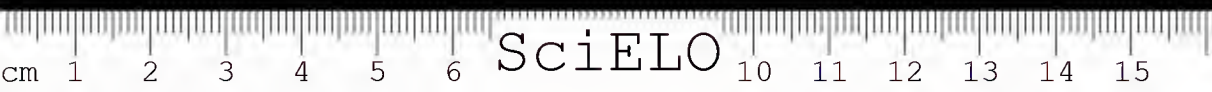
44. SOME ACTIONS OF SNAKE VENOM ON MITOCHONDRIA

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Introduction

The first indication of lipolytic activity of snake venom came from the work of Lüdecke (1) who showed that venom reacted with lecithin of blood plasma to produce a hemolytic agent. The first demonstration of the strong inhibition of electron transport came from the work of Ghosh and Chatterjee (2) who showed an inhibition with very dilute snake venoms acting on pigeon-brain cells over a 1½ hour period prior to testing for electron transport capability.

Nygaard and Sumner (3) showed that inhibition by lecithinase A must be occurring at more than one site because inhibition increased with substrates entering at levels further away from oxygen.

Edwards and Ball (4) showed that exposure of a succinoxidase preparation to *Naja naja* venom produced an inhibition of succinate oxidation which was proportional to the fatty acid released by the venom and that unsaturated fatty acid added as the sodium salt caused a similar inhibition. However, apparently because of the inhibition produced by *Clostridium welchii* toxin (which contains a phospholipase that splits phosphorylcholine from lecithin) they concluded that phospholipid was required for electron transport. They showed that added lysolecithins did not inhibit.

Aravindakshan and Bragança (5) showed that intraperitoneal injections of *Naja naja* venom led to the isolation of partially uncoupled rat liver and brain mitochondria.

Petrushka, Quastel and Scholefield (6) showed uncoupling activity of boiled *Naja naja* venom was reversible by phospholipid. Since Marsden and Reid (7) had reported the appearance of myoglobin in the urine of people envenomated by *Enhydrina schistosa*, we decided to try to release the cytochromes from mitochondria by use of *E. schistosa* venom (8). Although a slight release occurred, we became more interested in the actions on mitochondrial swelling and energy transformation. In a study made with *Bungarus fasciatus* venom (9), we demonstrated 1) that low levels of the venom (5-10 μg/mg protein) produced uncoupling of oxidative phosphorylation after 1-2 minutes exposure and 2) that the reduction of ADP*/O ratios (an index of the efficiency of the transformation of the energy in reduced coenzymes into terminal phosphate bond energy of ATP) paralleled the increase in venom (1-3 μg/mg protein) used to treat rat liver mitochondria.

If larger amounts of venom were used, the added ADP inhibited respiration rather than stimulated respiration. A similar inhibitory action of ADP in aged mitochondrial preparations has been termed reverse acceptor control by Leninger and Gregg (10). The inhibition of DPNH linked substrates was stronger than that for succinate oxidation (9). However, reversal of electron transport was possible in the presence of reverse acceptor control (9).

B. fasciatus venom boiled for 20 minutes at pH 5.9 to produce a phospholipase A preparation (11) caused uncoupling but not reverse acceptor control (9).

Since serum albumin (1% final concentration) reversed the uncoupling activity (9) and serum albumin had shown a similar reversal of the uncoupling activity caused by fatty acids present in mitochrome preparations (12), it was considered probable that the release of fatty acids was responsible for the venom action. In fact, the addition of neutral triglycerides to mitochondria during venom treatment greatly enhanced the inhibition of mitochondrial energy transformations, while addition of triglyceride to control mitochondria did not affect the energy transformations.

Several workers have reported that fatty acids cause either inhibition of respiration (13) or uncoupling of oxidative phosphorylation (12, 14).

A study of the venoms from a number of elapids showed that *Bungarus fasciatus, Micrurus fulvius, Naja naja* and *Walterinnesia aegyptia* venoms all showed uncoupling and reverse acceptor control activities, while *Denisonia pallidiceps* venom did not show either activity. None of three VIPERINAE tested had either activity. Of ten CROTALINAE tested, *Agkistrodon piscivorus* was highly active in both activities studied and *A. bilineatus* and *Bothrops jararaca* produced moderate uncoupling of mitochondria (15).

At the present time we are attempting to isolate the factor(s) in *Bungarus fasciatus* venom responsible for the production of the uncoupling and/or reverse acceptor control activity on incubation with mitochondria.

Methods

All reagents were obtained commercially and were of the highest purity available. *B. fasciatus* venom, collected in our laboratory, was stored as follows: unpooled, freshly collected venom was frozen immediately in an alcohol-dry ice mixture and either stored in a dessicator at −20°C (hereafter designated as frozen venom) or lyophilized in a Virtis Centrifugal Bio-Dryer and stored in a dessicator at −20°C (hereafter designated as lyophilized venom). Alternatively, freshly collected venom was stored in a dessicator at room temperature (hereafter designated as dessicated venom). Fresh venom (hereafter designated as fresh venom) was collected and used immediately. Commercial krait venom (Pure Toxin, hereafter designated as commercial venom) purchased as a lyophilized powder from Miami Serpentarium was stored at −20°C and reconstituted to a 10% solution in 0.05 M potassium phosphate, pH 7.4.

Horizontal starch gel electrophoresis was carried out according to the procedure of Smithies (16) using the discontinuous tris-citrate buffer system of Poulik (17). Gels were stained with Amidoschwarz 10B (National Biological Stain Color Index 20470). Concentrated staining solution contained 0.5 grams

Amidoschwarz in 100 ml of an acetic acid-ethanol-water solution 1:4:5. Dilute staining solution consisted of one part concentrated staining solution diluted with two parts acetic acid-ethanol-water solution 1:4:5.

In fractionation experiments, milking was carried out at 4°C. Elution of the components of fresh *B. fasciatus* venom after starch gel electrophoresis was according to the method of Master and Rao (18) with the following modification: after electrophoresis the starch gel was cut lengthwise into two halves. One half was stained with Amidoschwarz to determine the degree of migration of each of the components. A record of each pattern was made with a Polariod MP-3 camera. Photographs were taken on Polariod Land Film Packets, Type 55 P/N (Polariod Corp., Cambridge, Mass.) using a Tiffen Photar Filter (No. 8, Yellow 2).

The second half of the starch gel was then cut into strips according to the pattern indicated in the stained half. Isolated fractions were tested for uncoupling and reverse acceptor control activities with rat liver homogenates prepared in a mannitol-sucrose-EDTA medium as described by Ziegler *et* al. (19). Oxygen consumption was measured with a sealed oxygen electrode (20). Polarization of the Clark electrode and recording of the changes in oxygen tension was provided by a polarograph (Gilson Medical Electronics, Middleton, Wisc., Model K).

Protease activity was determined by the degree of clot lysis in a system consisting of 0.15 ml Bovine fibrinogen, 5 mg/ml (Armour), 0.05 ml of the venom fraction to be tested and 0.05 ml thrombin (citrate thrombin approximately 1.2 units). Protein concentration was determined using Haas' modification (21) of Lowry's method (22).

Results and discussion

In earlier work with *Bungarus fasciatus* venom (23), we had experienced difficulty with the stability of lyophilized venom, reconstituted with distilled water, when held at 2°C or when held in the frozen state at −16°C. Loss of production of reverse acceptor control activity occurred more rapidly than loss of uncoupling activity. Formation of a white precipitate accompanied the loss of activity (24). Because of the instability of the factors being studied in whole venom, we have avoided pooling of venom, preferring to use the venom from a single snake in a given experiment and/or storage procedure. We have repeated each experiment and/or storage procedure a number of times using venom from different snakes to insure that the typical results presented are not the result of individual variation.

In the initial studies of the separation of *B. fasciatus* venom by starch gel electrophoresis, either frozen or lyophilized venom was used to test the procedures for electrophoresis and staining. Since the patterns for the two storage forms differed, a systematic study of the effect of storage conditions on the electrophoretic patterns was undertaken.

When compared to fresh venom, frozen venom consistently showed a loss of all or a major portion of the fourth band from the cathode end and the presence of a lightly staining band ahead of the first band from the cathode end (the latter band is not always visible in photographs of the patterns).

Venom dessicated over silica gel at room temperature gave a pattern in which the protein bands present are shifted in relation to the patterns of fresh venom (Fig. 1).

The pattern of lyophilized venom appears to be closely related to that of fresh venom except for the absence of the first band migrating towards the cathode (Fig. 1).

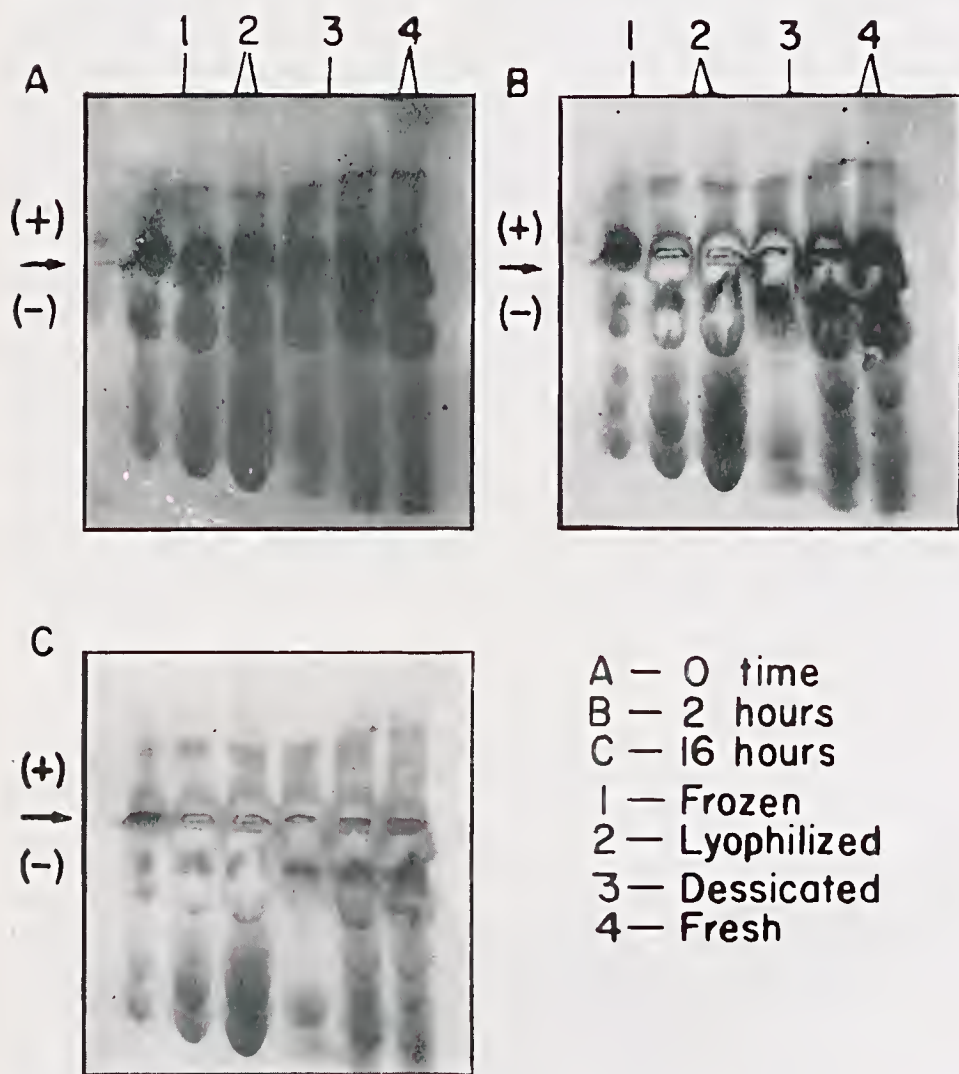



Fig. 1 — Variation in starch gel electrophoresis pattern of *Bungarus fasciatus* venom with different methods of storage. Electrophoresis was carried out at 4°C, for 9 hours, 300 volts. Starch gel was stained with concentrated Amidoschwarz 10B, prepared as described in METHODS, and washed in 95% ethanol-5% acetic acid, 1:1, and/or distilled water for time periods indicated.

A prominent feature of the venom electrophoretic patterns is the occurrence of "white spots", i.e., areas which originally stain with either dilute or concentrated dye but which wash free of dye rapidly in the patterns stained with dilute dye and more slowly in patterns stained with concentrated dye. These areas ap-

pear white because dye is bound less strongly than by the starch gel. In starch gel electrophoresis patterns of fractions in the preparation of bovine prothrombin, similar "white spots" were shown to be due to the presence of sulfated polysaccharides (25). However in this study the "white spots" occurred on the anode side of the point of application and were stainable with Alcian Blue and Mucicarmin. In our study the "white spots" are not stainable with either Mucicarmin or Alcian Blue and are on the cathode side of the point of application.

A sample of commercial *B. fasciatus* venom (Miami Serpentarium) was totally inactive in the mitochondrial test system and gave an atypical electrophoretic pattern in which several bands were faint or missing (Fig. 2).

CONCENTRATED
16 HOURS

1 — Commercial 2 — Lyophilized 3 — Frozen 4 — Blank

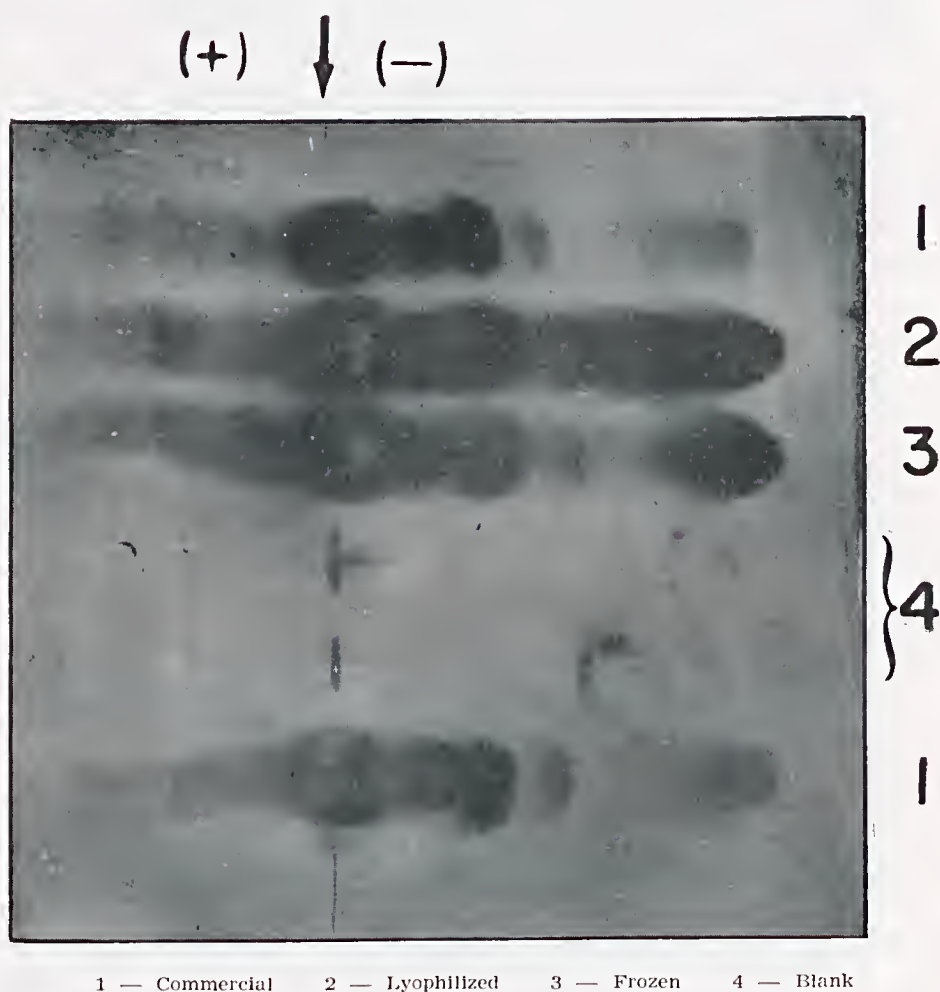
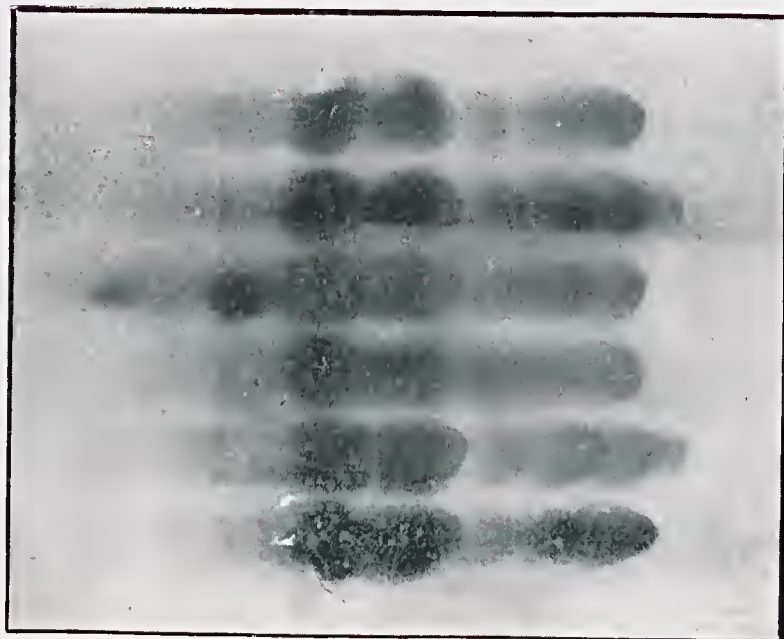



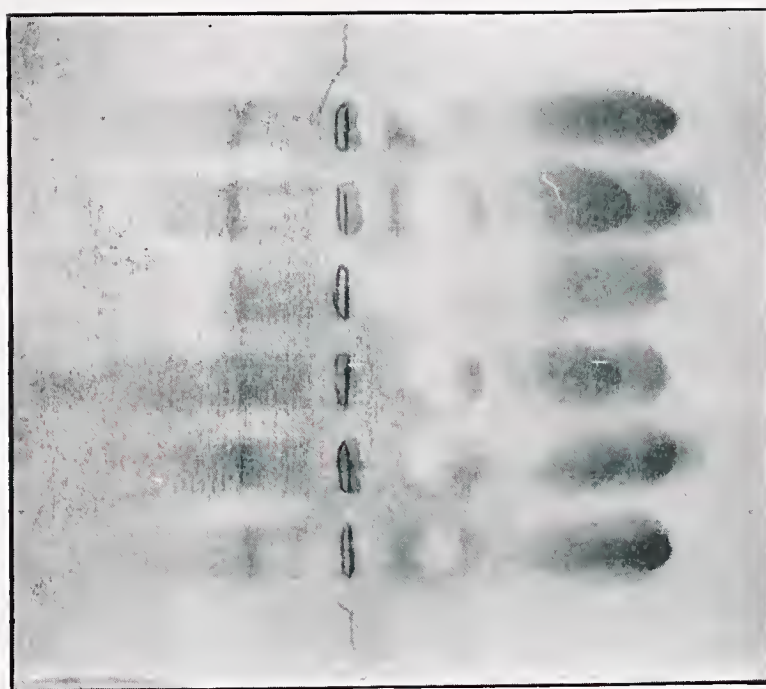
Fig. 2 — Starch gel electrophoresis pattern of commercial *Bungarus fasciatus* venom, 10% solution in 0.05 M potassium phosphate, pH 7.4. Frozen and lyophilized venom patterns shown for comparison. Electrophoresis and staining conditions same as in Fig. 1. Destaining time (in distilled water): 16 hours.

O TIME

CONCENTRATED

1 2 3 4 2 1

(+) → (−)

DILUTE

1 2 4 3 2 1

(+) → (−)

1 — Fresh 2 — Frozen 3 — Lyophilized 4 — Dessicated

Fig. 3a

Fig. 3b

1 — Fresh 2 — Frozen 3 — Lyophilized 4 — Dessicated

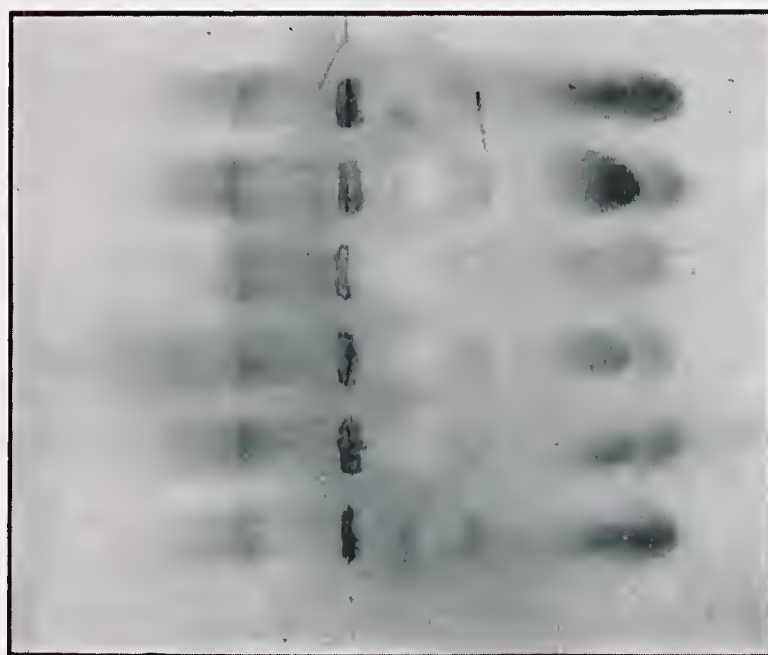
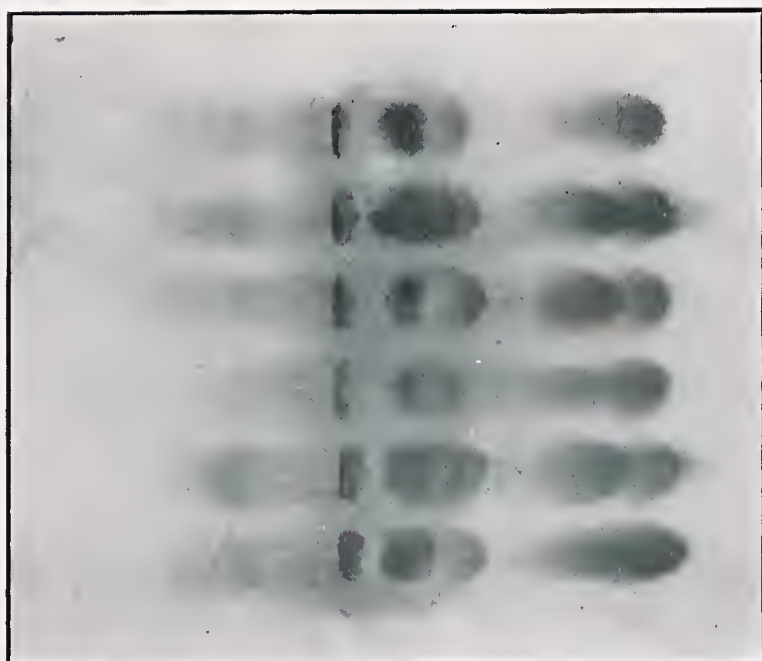



Fig. 3c

1 — Fresh 2 — Frozen 3 — Lyophilized 4 — Dessicated

Figs. 3a, b c — Variation of starch gel electrophoresis pattern of *Bungarus fasciatus* venom, stored as indicated, with either concentrated or dilute Amidoschwarz 10B (prepared as described in METHODS). Electrophoresis conditions same as in Fig. 1. Destaining times (in distilled water) as indicated. Note appearance of "white spots" which migrate toward the cathode and become more prominent with increased destaining time.

Uncoupling	+	+	+	+	+	+	−	−	−	−
Reverse Acceptor Control	−	−	+	+	−	−	−	−	−	−
Protease	?	−	−	−	?	?	+	+	+	+
Protein (mg/ml)	0.58	0.90	0.90	1.44	1.10	1.89	0.36	0.35	0.39	0.39

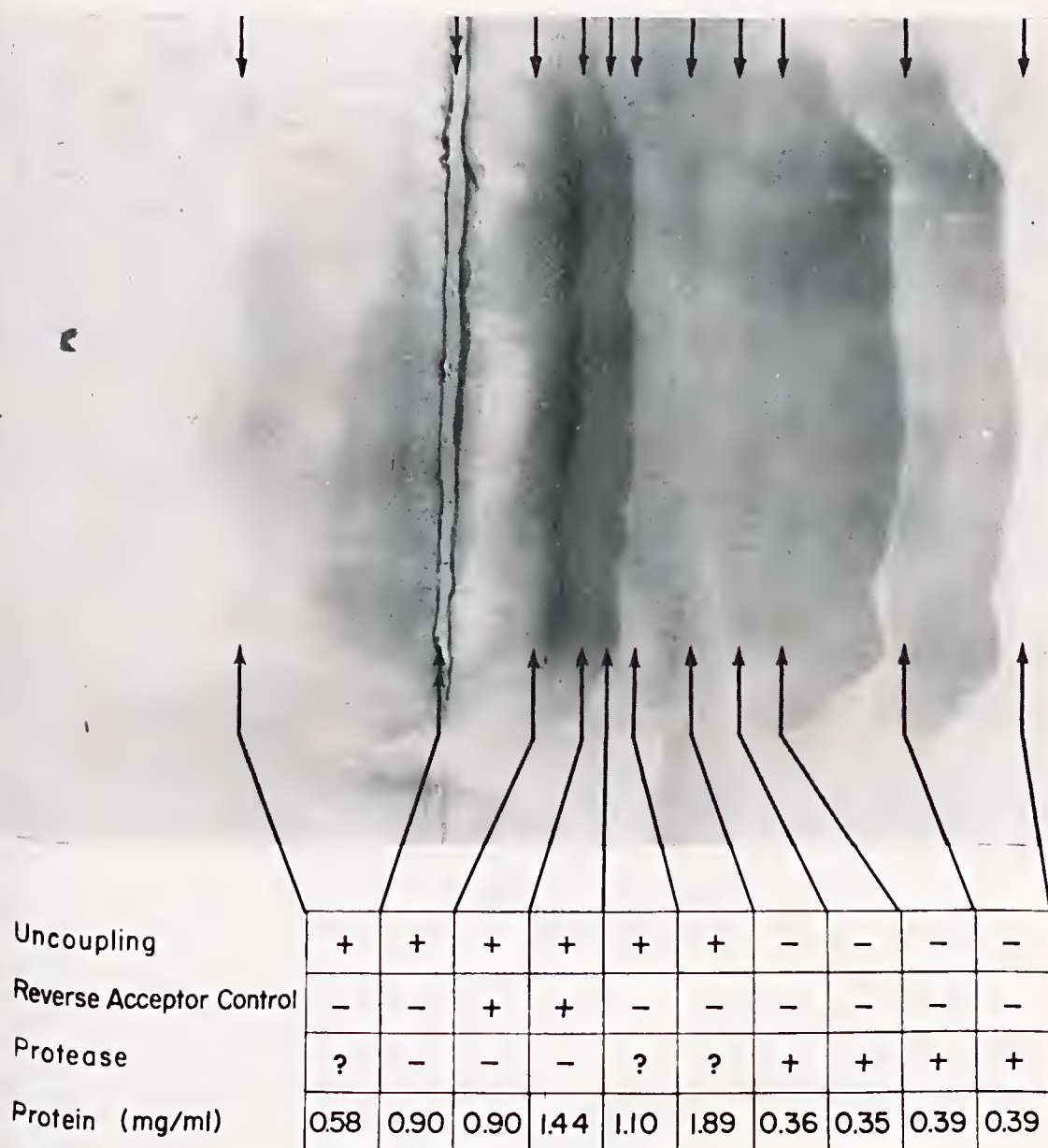



Fig. 4 — Typical starch gel electrophoresis pattern obtained in fractionation experiment with *Bungarus fasciatus* venom. Milking was carried out at 4°C and electrophoresis and staining conditions were the same as in Fig. 1. The unstained half of the starch gel was cut as indicated by the single headed arrows; double headed arrow marks the point of sample application. Uncoupling, reverse acceptor control, protease and protein were determined as described in METHODS.

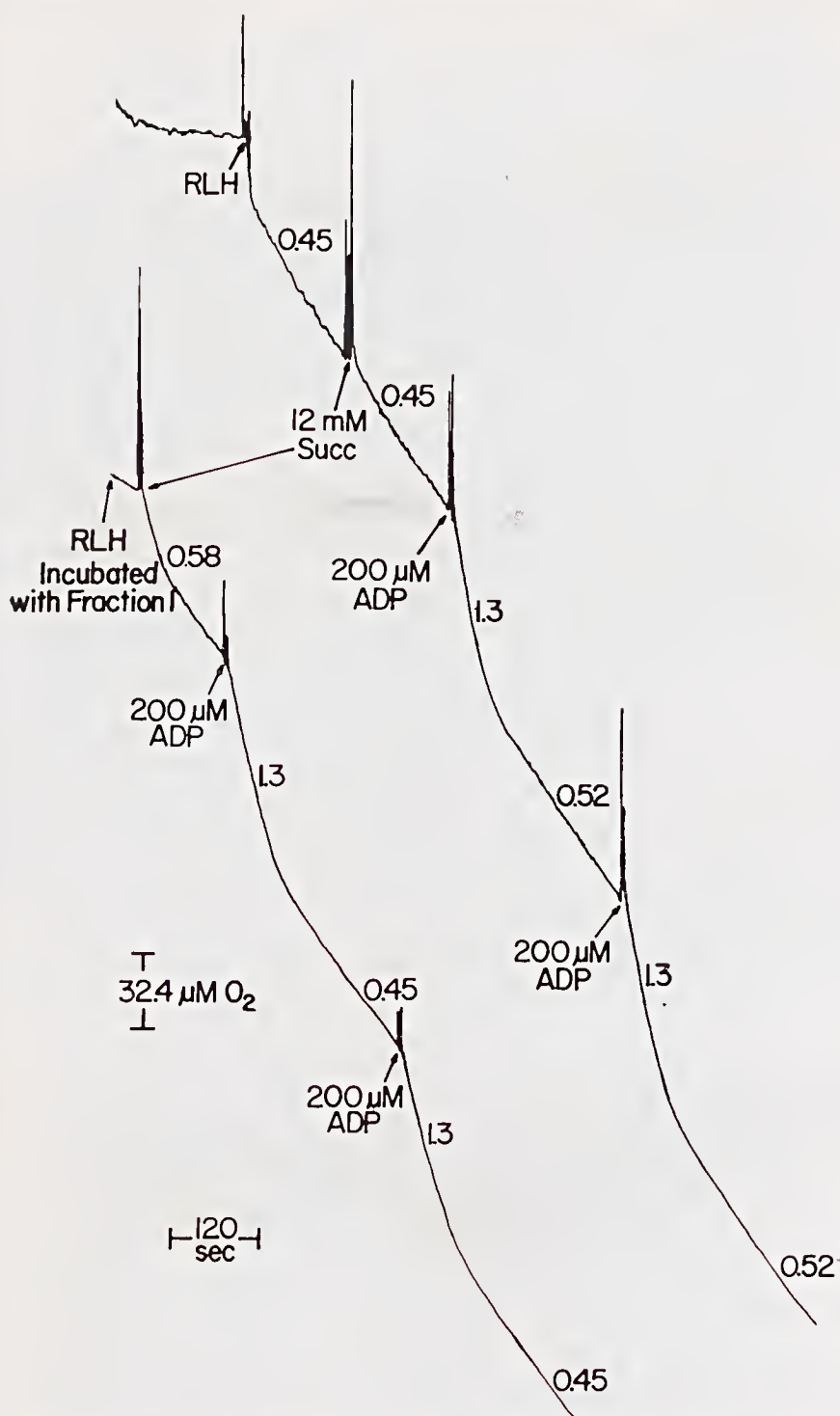



Fig. 5 — Effect of an inactive fraction (Fraction 1) of *Bungarus fasciatus* venom on a rat liver homogenate system. Top curve, a control experiment, shows stimulation of succinate oxidation by ADP in a rat liver homogenate, temperature 25°C, pH 7.4. The numbers above the curves indicate respiratory activity represented as $\mu M\ O_2/sec$. Concentrations of reagents added are indicated below the curves. The respiratory vessel contained a total volume of 2.5 ml. Bottom curve shows respiratory activity following 5 minutes incubation of rat liver homogenate with Fraction 1.

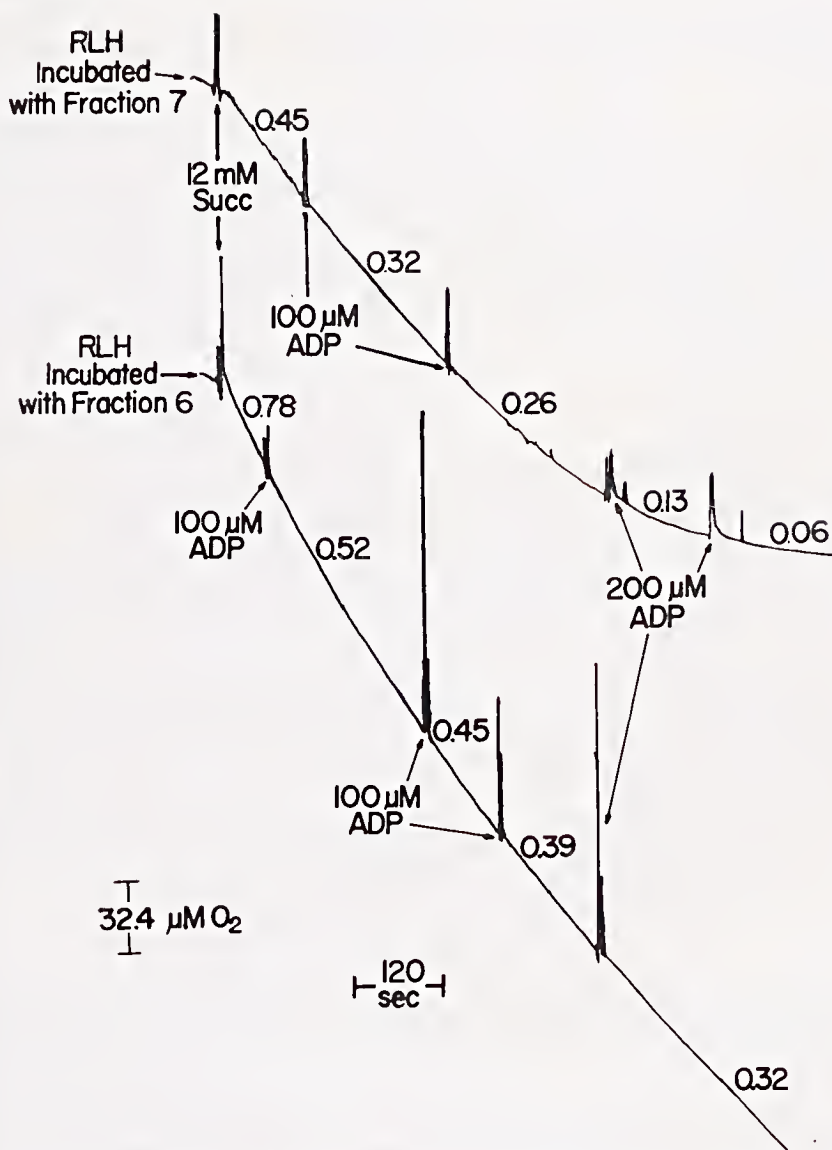



Fig. 6 — Effect of two active fractions (Fractions 6 and 7) of *Bungarus fasciatus* venom on a rat liver homogenate system. Reaction conditions and labeling as in Fig. 5. Fraction 6 shows uncoupling activity; Fraction 7 shows reverse acceptor control.

We have found the use of both concentrated and dilute dye, one on each half of the same starch gel pattern, useful in distinguishing fine details of the patterns. The following of the changes in intensity of the bands during washing with ethanol-acetic acid and/or distilled water is also useful (Fig. 3 a, b, c). There is a difference in the appearance of the "white spots" after 18 hours of washing in patterns stained with dilute and concentrated dye and a disappearance of the fastest moving bands in the frozen venom pattern with washing of the

pattern stained with dilute dye. Since the pattern is restored by restaining, the washing out reflects a difference in dye binding of the proteins of the different bands.

In view of the variation in patterns with storage, in venom fractionation experiments, snakes were milked in a 4°C cold room where the electrophoresis apparatus was preassembled and ready for application of the venom. Fig. 4 shows the typical results of a fractionation experiment. Uncoupling activity occurred in all but the leading four bands. Reverse acceptor control activity occurred in the seventh and eighth bands from the leading edge of the cathode portion of the pattern. It should be noted that the intensity of staining varies considerably in relation to the protein concentration as determined by the modified Lowry procedure.

Protease activity, as measured by degree of lysis of clotting system constituents, was limited to the first four bands. In one experiment, those isolated fractions which originally showed good uncoupling and reverse acceptor control activities retained these activities after more than 48 hours at 2°C in contrast to whole venom which showed total loss of activity in 24 hours or less.

Electrode tracings are shown for fractions having no activity (Fraction 1) and for fractions having uncoupling and reverse acceptor control activities (Fractions 6 and 7 respectively) on incubation with mitochondria (Figs. 5 and 6). Repetition of the fractionation experiment has occasionally shown the appearance of uncoupling and reverse acceptor control activities in bands adjacent (toward the cathode) to those shown in Fig. 4. Such overlapping of activities was attributed to the uneven migration pattern which makes it difficult to accurately measure and cut individual bands for isolation.

The question of whether the reverse acceptor control and uncoupling activities are due to one and the same enzyme or to a group of enzymes is being examined by heat denaturation and substrate specificity studies.

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Discussion

E. A. Zeller: "I stress the importance of clear-cut demonstration that there are very labile components present in snake venom. Do mitochondria obtained from both sexes show any difference in response to snake venoms?"

W. B. Elliott: "We have tried the venoms of four true vipers (VIPERINAE), *Echis carinatus*, *Bitis gabonica*, *Bitis lachesis* and *Eristocophis macmahonii*, none of which caused uncoupling of mitochondrial oxidative phosphorylation or reverse acceptor control. Of several CROTALINAE venoms studied, only *Agkistrodon piscivorus* showed high activity of both types."

45. THE VENOMS OF AMPHIBIANS

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The cutaneous secretions of amphibians contain an amazing variety of compounds of diverse pharmacological activities (Table I and Fig. 1). These include simple biogenic amines, peptides, steroids and steroidal alkaloids. Their pharmacological activity encompasses cardio-, myo-, and neurotoxins, cholinomimetic and sympathomimetic agents, local vasoconstrictors and hypotensive agents and even one of the most potent hallucinogens known, 0-methylbufotenine. Among these compounds are some of the most powerful venoms known.

TABLE I — TOXIC SUBSTANCES ISOLATED FROM AMPHIBIANS *

Substances	MLD (μg/kg)	Source	Class of Compound	Pharmacological activity
Batrachotoxin	2	Frog: *Phyllobates bicolor*	Steroidal Alkaloid	Cardio- and neurotoxin
Tarichatoxin (Tetrodotoxin)	8	Newt: *Taricha torosa*	Guanidine Derivative	Neurotoxin
Samandarine	300	Salamander: *Salamandra maculosa*	Steroidal Alkaloid	Central Convulsant
Bufotoxin	400	Toad: *Bufo vulgaris*	Steroid	Cardiotoxin
Dehydrobufotenine	6000	Toad: *Bufo marinus*	Indole	Convulsant
Leptodactyline	7500	Frog: *Leptodactylus pentodactylus*	Phenolic amine	Cholinomimetic Agent
0-Methylbufotenine	75000	Toad: *Bufo alvarius*	Indole	Hallucinogen

* For comparison the MLD of strychnine is 500, of d-Tubocurarine is 500 and of muscarin is 750.

The function of these compounds in amphibians may involve active defense or passive protection. In certain cases, as in toads of the genus *Bufo*, the secretions are actively ejected as a defense against certain enemies, such as dogs and other carnivores. Other amphibians, for example, the European *Salamandra maculosa*, make passive use of skin secretions as a defense against predators. The high toxicity of these secretions need not be viewed in terms of defense only. Some of these compounds may have physiological functions in the skin of the amphibian.

BUFOTENINE

DEHYDROBUFOTENINE

CANDICINE

EPINEPHRINE

SPINCEAMINE

BUFOTALIN

CARNOSINE

TARICHATOXIN (TETRODOTOXIN)

SAMANDARINE

Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

BRADYKININ
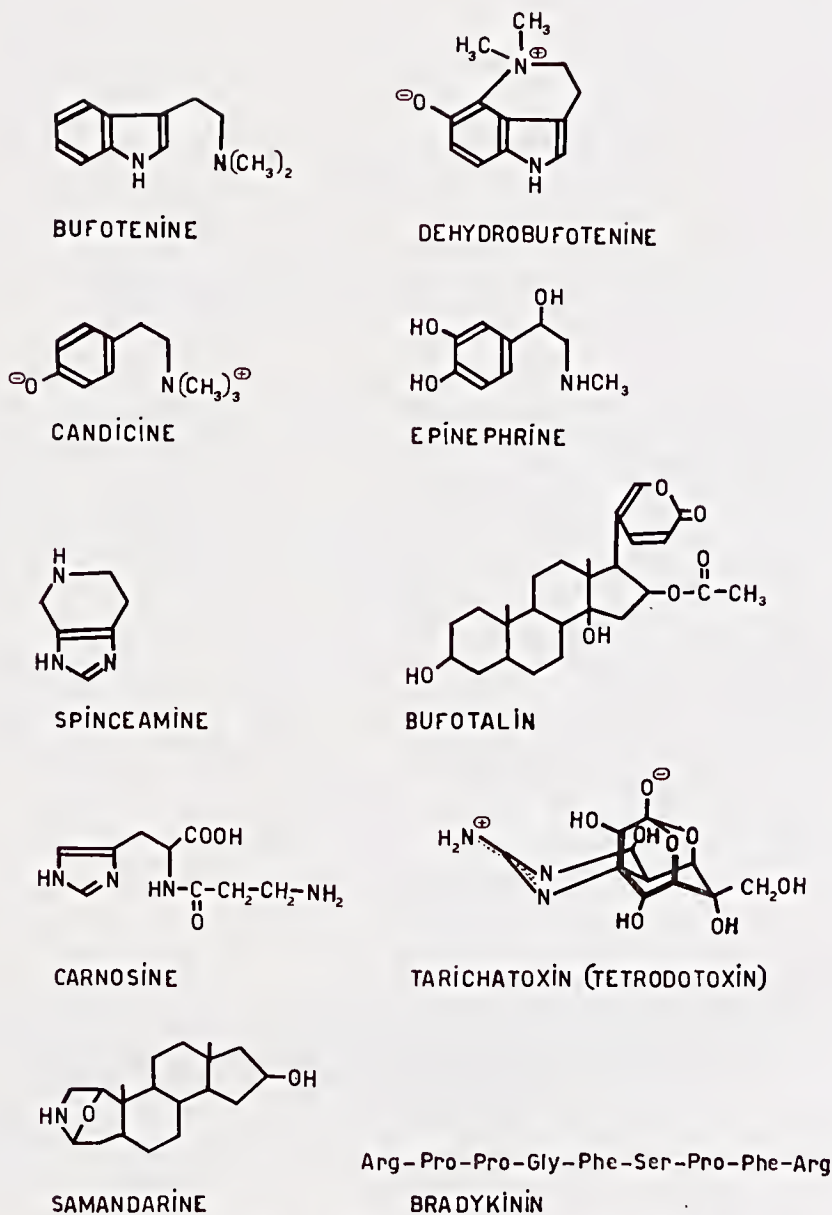
Fig. 1 — Representative Pharmacologically Active Compounds Isolated from Amphibian Skins.

Both *simple biogenic amines* and *complex bufodienolides* occur together in the parotid gland secretions of toads of the genus, *Bufo* and have been the subject of intensive investigations. Indolealkylamines such as *serotonin*, *N-methyl-serotonine*, *bufotenine*, *bufotenidine* and *5-methoxy-N,N-dimethyltryptamine* (1) have been isolated from toads of this genus. They possess a variety of activities and in-

clude vasoconstrictors, cholinomimetics and hallucinogens. The novel biosynthetic conversion of bufotenine to the *tricyclic dehydrobufotenine* in *Bufo marinus* is currently under study in our laboratories (S. Senoh, J. Daly and B. Witkop). Also present in the glands are various sympathomimetic amines such as *dopamine*, *norepinephrine*, and *epinephrine* (2).

Frogs of the tropical American genus, *Leptodactylus* contain three types of biogenic amines (3): the *indolealkylamines*, such as *serotonin* and *bufotenidine*, the phenolic amines, such as *tyramine*, *candicine* and *leptodactyline*, and the *imidazolealkylamines*, such as *histamine* and *spinceamine*. Candicine and leptodactyline are nicotine-like in action, while histamine is a local irritant. Spinceamine has no pronounced pharmacological activity.

Examination of 7 species of *Puerto Rican frogs* of the related genus *Eleutherodactylus* did not reveal significant quantities of any of these biogenic amines. A large amount (0.2-1.2 mg/g skin) of an amino acid which gave a Pauli positive color reaction typical of an imidazole was found in all these frogs (J. W. Daly and H. Heathwole, in press). This amino acid was not histidine and was at first thought to be spinacine, the amino acid precursor of the amine, spinceamine.

Chemical studies proved, however, that the material was the *dipeptide, carnosine* (*β-alanylhistidine*) previously known only from the muscle of certain vertebrates. Carnosine has no known physiological or pharmacological activity and certainly is not present as a protective poison in these frogs.

Other *peptides of higher molecular weight* have been found in a variety of amphibians (4, 5): *Bradykinin* and *physalaemin*, are *potent hypotensive agents*, while the *tryptokinins* have no known activity.

Completely different in structure is *tetrodotoxin* (*tarichatoxin*), the *extremely potent neurotoxin* from newts of the genus *Taricha* (6). This substance is reported only in newts and in puffer fish of the genera *Sphoeroides* and *Arothron*, an unusual case of biochemical disjunction.

Amphibians also contain lipid soluble poisons in their skin. These include the great variety of *bufogenins* which occur only in toads of the genus *Bufo* and which are *extremely active cardiotoxins* and *local anesthetics*. Various *steroidal alkaloids* are also known from amphibians. *Samandarine*, *samandarone* and related compounds occur in the skin of salamanders of the genus *Salamandra* (7) along with *hemolytic proteins*. Samandarine has also been reported recently from an Australian anuran of the genus *Pseudophryne* (8). Samandarine is a *centrally active convulsant* of high toxicity.

Steroidal alkaloids have now been found to be present in the skins of tropical American dendrobatid frogs of the genera, *Phyllobates* and *Dendrobates*. In particular the skin of the *poison arrow frog of Colombia*, known as Kokoi' to the natives of the Choco' of that country, contains an extremely poisonous alkaloid. It has been used for centuries to poison blow gun darts for hunting small game by Indians of this region.

The frog, which has been provisionally identified as *Phyllobates bicolor*, is tiny and contains only minute amounts of the venom which was named *batrachotoxin* (9, 10). A single frog contains only 40-80 micrograms of batrachotoxin which is, however, sufficient to kill 2-4,000 mice on intravenous injection. On oral administration, batrachotoxin is 60 to 100 times less toxic.

Three expeditions to the Choco' Jungle of western Colombia in 1961, 1964 and 1966, under the able leadership of explorer-zoologist Mrs. Martè Latham, have netted over 7,000 frogs.

Two color varieties of the Kokoi' are present in this region of Colombia. The main difference is in the width and color of the dorsolateral stripes. These are narrow and bright yellow in the frog from lower elevations above *Playa de Oro* on the *Rio San Juan*, while at higher elevations in the same watershed, a slightly larger frog with yellow-orange to red-orange stripes is found. Often the dorsolateral stripes merge and cover the entire back of this mountain variety. Both varieties contain batrachotoxin in comparable quantities. The *frogs appear to be quite resistant to large doses of their own venom.*

The method of purification of batrachotoxin has now been simplified as follows: To a methanol extract of the skins is added 1 volume of water; the alkaloids are then extracted into chloroform. The basic alkaloids are removed from the chloroform by extraction into 0.1 N hydrochloric acid.

The aqueous acid is adjusted to pH 8.5 and reextracted with chloroform. The chloroform extract is concentrated *in vacuo* and the final purification makes use of thin-layer chromatography as described previously (9). All steps must be carried out at 5°C to prevent large losses of activity. Studies on the structure of batrachotoxin have been hindered by this instability and by the paucity of material available for study, even from 7,000 frogs.

Batrachotoxin is a weak base of pH 7.5 as measured by partition coefficients. Its ultraviolet spectrum shows only end absorption indicating a lack of conjugated double bonds. The infrared spectrum indicates hydroxyl groups. An intense band at 1690 cm^{-1} could be either due to a carbonyl group or to a vinyl ether, but the usual tests for a carbonyl function are negative in batrachotoxin and the optical rotary dispersion curve displays no Cotton effect in the region where steroidal ketones or aldehydes exhibit such effects. A strong absorption in the infrared spectrum at 1250 cm^{-1} also suggests not a carbonyl group, but a vinyl ether.

The nuclear magnetic resonance spectrum indicates a quaternary methyl group, a methyl group on a tertiary carbon attached to a hetero-atom and 3 hydrogens at low field which are assigned to a carbinolamine (-CH(O-)(N-)) group, a vinyl ether (C = C(H)(O)) group and an olefinic proton in proximity to a hetero atom.

The *high resolution mass spectrum* of batrachotoxin gave an *empirical formula* of $C_{24}H_{33}NO_4$ which indicates the presence of 9 rings or double bonds. The loss of CHO from the parent ion is evidence for a potential aldehyde group. The n.m.r. spectrum also indicates a small amount of aldehyde and amine in equilibrium with the carbinol amine function. Fragmentation of batrachotoxin, with loss of C_4H_7N to form the ion $C_{20}H_{26}O_4^{\oplus}$, suggests that, instead of a carbinolamine, a carbinol amine ether is present in batrachotoxin. The ion $C_{20}H_{26}O_4^{\oplus}$ loses in succession 3 molecules of water to form $C_{20}H_{20}O^{\oplus}$. These transformations are confirmed by the presence of the corresponding meta-stable peaks. The ion $C_{20}H_{20}^{\oplus}$ may then lose an aldehyde group (CHO) to form $C_{19}H_{19}^{\oplus}$. This is

good evidence for a continuous carbon skeleton of at least 19 atoms. The low molecular weight fragments, $C_8H_{11}NO_2^{\oplus}$, $C_7H_8NO_2^{\oplus}$ and $C_4H_{10}NO^{\oplus}$ lead to the assumption that a methyl group, the nitrogen and two of the oxygens are within 7 carbons of each other and that a methyl group, the nitrogen and one oxygen are with 3 carbons of each other.

The mass spectrum of batrachotoxin was also measured after exchange with D_2O. Two exchangeable hydrogens were found. One was associated with the $C_7H_9NO_2^{\oplus}$ fragment. The nitrogen atom did not appear to have an exchangeable hydrogen.

Chemically batrachotoxin gives a *positive, immediate Ehrlichs test* which must be due to a *potential pyrrole group* in its structure.

On the basis of other reactions and the spectral data, batrachotoxin is a 24 carbon modified steroid with a potential 5 membered pyrrole ring that contains a tertiary nitrogen as part of a carbinol amine-vinyl ether group and a double bond. Within two carbon atoms of this ring is one of the two alcohol groups. The remainder of the molecule contains the other two oxygen atoms, one in an alcohol group and one in an ether linkage. This part of the molecule probably contains a tetrasubstituted double bond.

The complete structure of batrachotoxin must now await further studies by n.m.r. and mass spectrometry on the catalytic and hydride reduction products and various other derivatives. Attempts are being made to prepare a crystalline derivative for analysis by X-ray crystallography .

Pharmacologically, batrachotoxin is the most toxic known non-protein material (MLD 1 μg/kg) with cardiotoxic, myotoxic and neurotoxic activities.

In rat diaphragm-phrenic nerve preparation, indirect stimulation is quickly blocked and direct stimulation more slowly. A powerful contracture of the muscle also develops. These events are irreversible.

In a sciatic-sartorius preparation (*Bufo marinus*), the action potential of the nerve is relatively unaffected at concentrations which decrease the action potential in the muscle. The muscle after complete blocking of its action potential can still respond to direct stimulation indicating a block in neuro-muscular transmission. *In vivo,* in cats and dogs, a dose of batrachotoxin (0.3-.5 μg/kg i.v.) which does not significantly effect the response of muscle to nerve stimulation did cause interference with conduction in the heart, extrasystoles and finally ventricular fibrillation and death. Little effect on blood pressure was noted.

Other dendrobatid frogs have now been examined for toxic alkaloids. These include *Dendrobates tinctorius* (2 color varieties, Playa de Oro, Colombia), *Phyllobates subpunctatus* (Bogotá, Colombia), *Phyllobates talamancae* (Panama), *Phyllobates pratti* (Panama), *Phyllobates lugubris* (Panama), *Dendrobates minutus* (Panama), *Dendrobates auratus* (Taboga, Panama). Of these species, only *Dendrobates auratus,* which is known as the poison arrow frog of Panama, the tiny *Dendrobates minutus,* and some of the color varieties of *Dendrobates pumilio* had appreciable toxicity in skin extracts. None of these could, however, be compared to the toxicity of the extremely poisonous Kokoi' of Colombia. No batrachotoxin could be detected in any of these other frogs so that the Kokoi' of Colombia appears at present to be quite unique in this respect.

These studies on Panamanian frogs are the result of a stimulating collaboration with Charles W. Myers of the Gorgas Memorial Laboratory, Panama. He has discovered that *Dendrobates pumilio,* a small red and black frog in large

areas of Central America explodes into a great number of variously colored island populations in the Bocas region of Panama. The coloration between populations is extremely varied both in the dorsal and ventral aspects. What factors have caused this diversification are at present unknown. Since warning coloration in amphibians is often assumed to be associated with venomous secretions, a *comparison of the toxicities of various populations with brightness of coloration* was carried out (J. W. Daly and C. W. Myers. In preparation).

No *correlation between color and toxicity* was found although both factors varied widely between populations. Certain brightly colored frogs were almost nontoxic while one dark-blue frog, very protectively colored, contained large amounts of venom. The toxicity was measured by subcutaneous injection in mice and also by visualization of toxic principles A and B on thin-layer chromatoplates. The two toxic principles were then isolated by alumina column chromatography and silica gel thin-layer chromatography. These two toxic principles were found by high resolution mass spectrometry to have empirical formulae of $C_{19}H_{33}NO_2$ and $C_{19}H_{33}NO_3$, respectively. The ultraviolet absorption spectra showed only end absorption. The infrared spectra showed no carbonyl, double bond or oxazolidine ring. The mass spectra provided evidence for 4 rings, a carbinolamine, and one or two hydroxyl groups. Both compound A and B formed 0-methyl ethers on treatment with methanolic hydrochloric acid. Compound A forms a 0,N-Diacetyl derivative. The foregoing data suggest that compounds A and B are related in structure to the salamander alkaloids. These studies were carried out on only about 1 mg of each compound and the final structural elucidation will require additional material.

A great variety of other amphibians are known to contain toxic substances in their skin secretions, and it appears that their investigation should be quite profitable in terms of discovery of novel chemical structures and compounds of high pharmacological activity.

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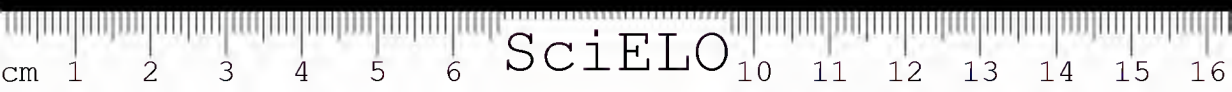
DISCUSSION

F. E. Russell: "Dr. Daly, in the slide showing the effects of the toxin on a Bulbring nerve-muscle preparation you noted that this demonstrated the neuromuscular blocking activity of the toxin. Did not the slide also show a reduction in the directly ilicited contractions which would certainly make it difficult to evaluate the neuromuscular blocking effect; and secondly, how can you be sure, in this preparation, that the principal effect is not on the nerve, rather than on the muscle?"

J. Daly: "I was not clear enough in explaining that in sciatic-nerve-Sartorius muscle preparation in *Bufo marinus,* that the nerve action potential was unaffected and the muscle action potential decreased. The muscle at this point still responded to direct stimulation thus indicating a block in neuromuscular transmission."

46. CARDIOTOXIC STEROIDS FROM TOADS

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Knowledge of the poisonousness of the toad goes back to antiquity. Physicians of the classic period mentioned in their writings medicines prepared from toads, and described their effect on the heart and respiration. In the seventeenth and eighteenth centuries dried toads were temporarily admitted as "bufones exsiccati" in the official pharmacopoeias and were used as diuretics against dropsy and other diseases, even before digitalis was introduced by Withering. In China and Japan the dried venomous secretion of the Chinese toad, formed into round, smooth, dark brown discs and known as Ch'an Su or Senso, is still used to-day against various diseases.

The skin glands of elliptical form, the so-called paratoids, contain the bulk of the venom and are located in the rear of the ear. A much smaller amount of venom is also secreted by the small verrucose skin glands that cover the whole back of the animal. In this secretion two groups of toxic substances are found together with other compounds.

1. *the heart-active principles,* representing steroid derivatives, usually known as *bufogenins* (bufagins), or systematically as bufadienolides, and *bufotoxins* (conjugates of the bufogenins with suberylarginine), which are primarily responsible for the pharmacological effect of the poisonous secretion, and

2. *the basic components* of the bufotenine type as well as the hormones adrenaline and noradrenaline.

Elucidation of the chemical structure of the bufogenins which are the subject of this paper has been started by H. Wieland and his associates in Germany, by Jensen and Chen in America, and by several groups in Japan such as Kotake and Kuwada, and Kondo and Ohno. But only during the last two decades unequivocal proof of the steroid nature of the bufogenins has been presented and the details of their structure completely elucidated.

On the following table all toad species are listed whose venomous secretion has been subjected to a closer chemical investigation.

The toad poisons can be obtained from the following sources: from the dried skins of the animals, from secretions of the skin glands which are stimulated by electrical irritation of the living animals, or best, from dried paratoid secretion. (In this connection it should be mentioned that the poisonous substances have also been detected and isolated in other parts of the body, e.g. in the blood, and the ovaries.) The collection of the venomous secretion from the living toads can be effected as follows: the animals are firmly held with one hand and pressed down on a board that is covered with a glass plate. The raised, oblong gland ac-

cumulations located behind the ear are held near their base, between thumb and forefinger and squeezed firmly, so that the milky secretion squirts onto the underside of the glass plate. The glass plates charged with the venom secretion are kept in a horizontal position (secretion on top) at room temperature without exposure to direct sunlight until the secretion is dry and can easily be scraped off. This slow drying probably permits the enzyme contained in the raw secretion to split the bufotoxins into bufogenins and suberylarginine. The above procedure can be repeated after a rest period of 30-40 minutes and yields again a considerable quantity of secretion. Afterwards the venom glands are completely empty and regeneration takes place after a period of 4 to 6 weeks. An excellent source for obtaining a series of bufogenins is Ch'an Su or Senso, mentioned above. This is still obtainable in large quantities from the pharmacies, for instance in Hong Kong and Tokyo.

SPECIES	SOURCE	Length of animals	Approx. amount of venom (dried) in mg per animal
Bufo alvarius GIRARD	Southern Arizona and Southern California to Mexico	80-165 mm	400
Bufo americanus HOLBROOK	Eastern part of North America from the Hudson Bay southward	54-110 mm	16
Bufo arenarum HENSEL (*arenarius* LUTZ)	Uruguay, Northern Argentina, Southern Brazil	75 mm	87
Bufo asper	Indonesia, Siam, Malayan Peninsula	260 mm	—
Bufo blombergi MYERS & FUNKHOUSER	Columbia	200 mm	1200
Bufo bufo bufo LINNAEUS = *Bufo vulgaris* LAURENTI	Europe, not including the Mediterranian, temperate zone of Asia	60-80 mm	13
Bufo bufo gargarizans = *Bufo gargarizans* CANTOR = *Bufo asiaticus*	China	75-114 mm	19
Bufo crucifer WIED 1821	Costline of Brazil and Argentina	up to 75 mm	18
Bufo formosus BOULENGER	Japan	125 mm	75
Bufo woodhausi fowleri HINKLEY	New England and New York south to Georgia and westward along the Great Lakes to Michigan. Along the gulf coast to central Texas	51-82 mm	14
Bufo granulosus SPIX subsp. *fernandeze* GALLARDO	East-north-eastern part of the South American continent from Panama to the southern part of Buenos Aires in Argentina	50-55 mm	—
Bufo ictericus SPIX 1824 = *Bufo marinus* BOULENGER, 1882, part.	Brazil	up to 140 mm	190

SPECIES	SOURCE	Length of animals	Approx. amount of venom (dried) in mg per animal
Bufo marinus (LINNAEUS 1758) = *Bufo marinus* (L.) SCHNEIDER	West Indies, Mexico, Central and South America	up to 200 mm	580
Bufo mauritanicus SCHLEGEL	Morocco, Algeria and Tunisia	122 mm	190
Bufo melanostictus	Southeast Asia, Indonesia	116 mm	90
Bufo paracnemis LUTZ	Guiana, Eastern Brazil	130-220 mm	240
Bufo peltocephalus TSCHUDI	Cuba	130 mm	120
Bufo quercicus HOLBROOK	North Carolina to Florida west from Louisiana	19-32 mm	2
Bufo regularis REUSS	Africa, wide spread	up to 136 mm	180
Bufo spinulosus WIEGMANN (=*B. chilensis* (TSCHUDI)	Chile, Peru	up to 100 mm	—
Bufo valliceps WIEGMANN	Louisiana, East and South Texas to New Mexico and Costa Rica	53-125 mm	18
Bufo viridis viridis LAURENTI	Europe, not including Iberian peninsula, North Africa, Near East, eastward to Mongolia, Tibet and Himalaya area	80-140 mm	27

For the isolation of the bufogenins from the dried paratoid secretion (e.g. from Ch'an Su) the finely pulverized (powdered) material is mixed with an equal volume (or more) of sand and extracted in a Soxhlet apparatus or in a percolator with chloroform. The yellowish-brown coloured extracts, containing the sterols and the bufogenins, are submitted to an initial purification in order to separate these two classes of compounds. The bufogenins so obtained are chromatographed on alumina or silica Gel. Thus, relatively easily and in a short time, the major bufogenins can be obtained in a crystalline state. However, those substances present in small or minute quantities may require further purification by chromatographic methods, for example partition chromatography, which may be used directly for separation of the crude venom, instead of absorption chromatography on alumina or silica Gel. Mixtures which are difficult to separate may be resolved by preparative paper chromatography or dispersion on long columns of silica Gel.

With the aid of Paper Chromatography or Thin Layer Chromatography it is possible in most cases to obtain an unequivocal identification of the bufogenins, thus enabling the chemist to analyse minute amounts of the venom. Furthermore, these methods are most reliable and easy for determining the homogeneity of the crystalline materials. The chromatograms are dried and the migrated substances can be detected by spraying with a solution of $SbCl_3$ in chloroform (20 gr/100 ml) and heating to about 80°-120° for several minutes. The dif-

ferent colours thus appearing in day- or ultraviolet light help further to characterize these substances. Since the bufogenins have a strong ultraviolet absorption at about 290-300 nm they can be located directly on the paper by a photocopy with filtred ultraviolet light, or even better with a monochromator. By this way 0.005 mg of a bufogenin can be detected.

The different colorations or the change of the colours which occur when the bufogenins are treated with strong acids are very useful in identification. It should be emphasized here that reliable results are only obtained if pure crystals are used and at the same time authentic substances are tested as well. With concentrated sulphuric acid or 84% sulphuric acid it is best to use white spot plates, but colour reactions with a solution of $SbCl_3$ in chloroform (20 gr/100 ml) are performed on filter paper. Only minute amounts of substances are needed (0.05 mg). The coloured spots should also be examined under ultraviolet light. The Liebermann colour reaction and its modifications have become obsolete.

The chemistry of the bufogenins

In contrast to the C_{23}-steroids of the digitalis and strophanthus type, the bufogenins are C_{24}-steroids. Their steroid nature was first deducted from dehydrogenation experiments: chrysene was obtained from bufotalin, and -methylcyclopenteno-phenanthrene from cinobufagin, cinobufotalin and marinobufagin. Direct transformation of a bufogenin into a steroid of known structure has not been achieved until 1939.

1) *The Lactone group*

An integrant part of all bufogenins is the lactone group. Wieland and Weyland first formulated this lactone group as five-membered on the basis of experiments with ozone (formation of formic acid). Later a structure with only one double bond in a six-membered lactone was proposed. In 1936 Wieland and associates correctly interpreted the ultraviolet spectrum [$\lambda_{max}^{alcohol}$ 290-300 nm ($\log \varepsilon = 3.75$)] of the bufogenins as derived from an α-pyrone structure. The same spectrum is found with coumalic acid methyl ester as well as with the scylla glycosides and their aglycones. The structure of the lactone ring is unambigously proved by the given spectral datas, by degradation experiments and also by synthesis.

2) *The Steroid ring skeleton*

Stereochemistry. The stereochemistry of the ring junctures at C-5, C-8, C-9, C-10, C-13, is the same for all naturally occurring bufogenins and corresponds to the ring junctures of the bile acids series, with the only exception of the C/D ring juncture, which is cis instead of trans.

3) *The Degradation*

Can be effected by different ways. By hydrogenolysis the bufogenins can be transformed into a bile acid. As relatively large amounts of substance are needed, this method has been given up. It has been shown that the best method

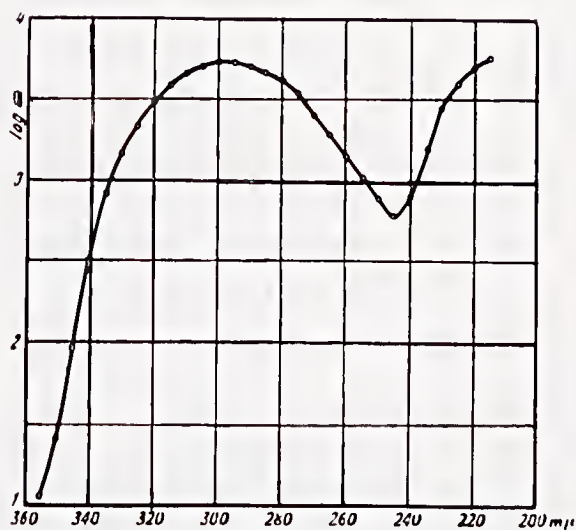
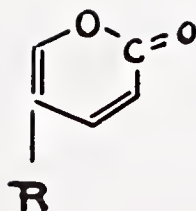



Fig. 1

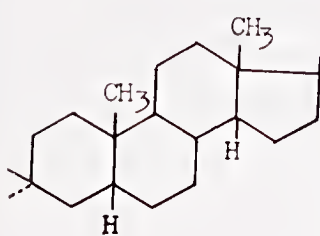
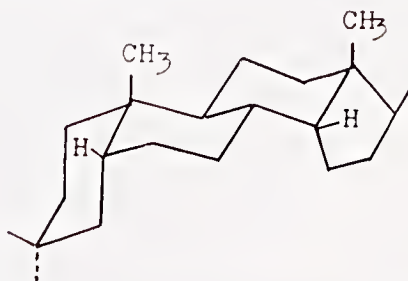
The Ring Skeleton of the Bufogenins

Projection Formula

Conformation Formula

Fig. 2

of degradation is the permanganate oxidation of the peracetylated bufogenins. By this method the corresponding etianic acids can be obtained in a relatively high yield (up to 50%).

3β-Hydroxycholanic acid

Bufalin

3β-Hydroxy-5β-etianic acid

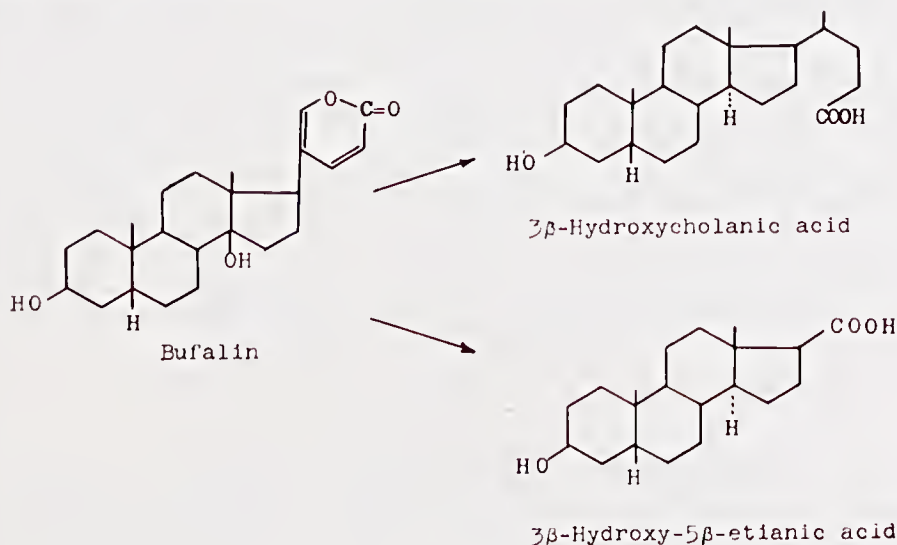



Fig. 3

4) *The Oxygen functions*

The bufogenins differ primarily in the number and position of the *hydroxyl groups*, which are scattered all over the skeleton. Each bufogenin has at least a secondary hydroxyl group at C-3 (recently 3-ketobufogenins have been isolated in minute quantities) and most bufogenins have as an integrant part a tertiary hydroxyl group at C-14 (similar to the cardio-active aglycones of the digitalis-strophanthus group). Those bufogenins carrying an *oxido group* at C-14/C-15 belong to a separate group. Additional hydroxyl groups are found at C-5, C-11, C-12, C-16 (mostly in acetylated form) and at C-19. A *keto group* was detected at C-12 and an *aldehyde group* at C-10.

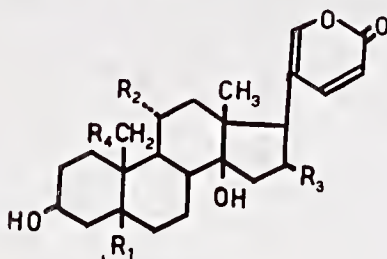



Fig. 4

R_1, R_2, R_3, R_4 = H = Bufalin
R_1, R_3, R_4 = H; R_2 = HO = Gamabufotalin
R_1, R_2, R_4 = H; R_3 = $OCOCH_3$ = Bufotalin
R_2, R_3, R_4 = H; R_1 = HO = Telocinobufagin
R_2, R_3 = H; R_1, R_4 = HO = Hellebrigenol

Arenobufagin

Hellebrigenin

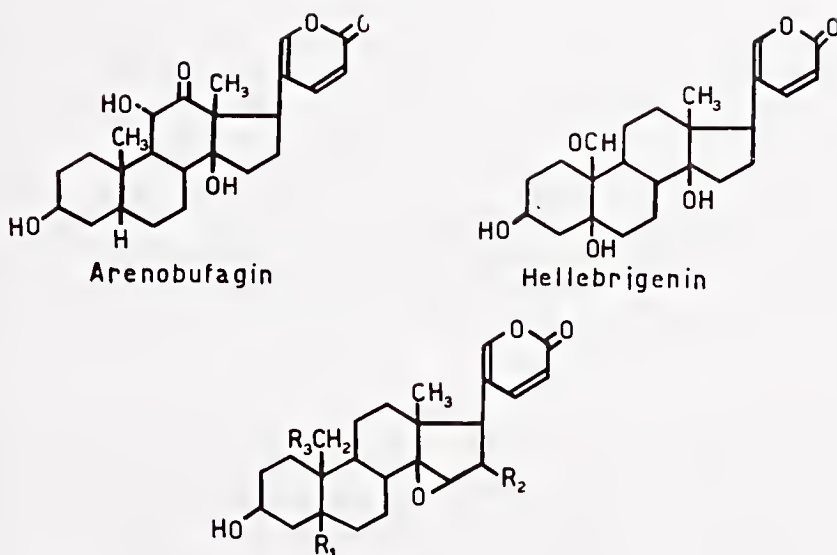



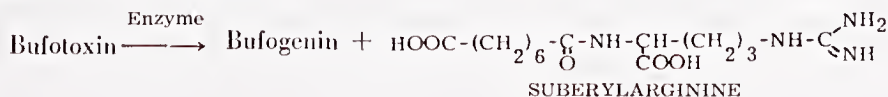
Fig. 5

R_1, R_2, R_3 = H = Resibufogenin
R_2, R_3 = H; R_1 = HO = Marinobufagin
R_1, R_3 = H; R_2 = $OCOCH_3$ = Cinobufagin
R_1 = H; R_2 = $OCOCH_3$; R_3 = HO = Cinobufaginol
R_1 = HO; R_2 = $OCOCH_3$; R_3 = H = Cinobufotalin

The bufotoxins

Toad venoms contain beside the bufogenins a further type of cardio-active substances, the so-called bufotoxins. These represent conjugates of bufogenins with suberylarginine and were isolated and investigated in a number of laboratories during the 1930's. With the exception of Wieland's bufotoxin, all are rather poorly characterized due to the fact that these substances are extremely difficult to obtain in homogeneous crystals. This is even the case when modern chromatographic methods are applied. No doubt the bufotoxins so far described are mixtures, and it seems very probable that Wieland's bufotoxin also was impure. Our assumption that the suberylarginine rest (in Wieland's Bufotoxin) is linked to the hydroxy group at C-14, seems, as Fieser already suggested, very unlikely. New investigations made in our laboratory tentatively confirm that the linkage point is at C-3.

$$\text{Bufotoxin} \xrightarrow{\text{Enzyme}} \text{Bufogenin} + HOOC\text{-}(CH_2)_6\text{-}\underset{O}{\underset{\|}{C}}\text{-}NH\text{-}\underset{COOH}{\underset{|}{CH}}\text{-}(CH_2)_3\text{-}NH\text{-}C\begin{matrix}NH_2\\NH\end{matrix}$$

SUBERYLARGININE

The biogenesis of the toad poisons

Very little is known about the biosynthetic pathway of the formation of the toad poisons. The close relationship in the structure of the bufadienolides and the bile acids indicate that these two types of steroids are formed from the same

basic substance. Tschesche and Korte assumed that the lactone ring of the bufadienolides is built up from oxalacetoacetic acid. The investigations so far undertaken with labelled substances, which could serve as building stones for the biosynthesis of the bufadienolides, have only shown, that radioactive carbonate, or sodium acetate-1-C^{14}, and sodium acetate-2-C^{14} are not incorporated. On the other hand, by feeding *B. marinus* with C^{14}-containing algae or by parenteral administration of cholesterol-4-C^{14} it was possible to obtain radioactive marinobufagin and marinobufotoxin, respectively. These results show that cholesterol is a precursor in the synthesis of the bufogenins and bufotoxins. This observation would suggest that cholesterol or a closely related compound is the major source of the cardiotonic sterols in the toad.

The synthesis of bufadienolides

While the synthesis of a cardenolide, i.e. Digitoxigenin was prepared only recently, the synthesis of a C_{24} steroid of the bufogenin type has been achieved in 1961 by Bertin and co-workers. But this lactone possessed neither the 14β-configuration nor the hydroxy group at C-14, which means that no "true" bufadienolide could be synthesized.

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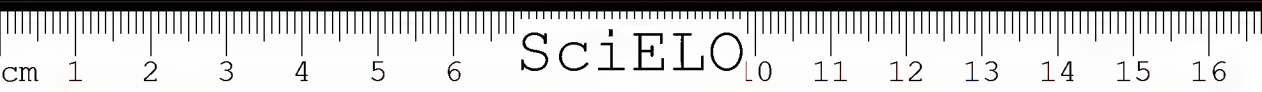
Discussion

E. A. Zeller: "In order to find out the hydroxyl group to which the suberylarginine is attached, enzymic studies with simple model substances and the enzyme present in crude venom preparation could be carried out. Did anybody try this way?"

K. Meyer: "The isolation of the enzyme was not yet tried."

47. WASP KININ

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During an examination for histamine-releasing activity in water extracts of dried venom apparatuses (sacs and glands) of *Vespa vulgaris*, Jaques and Schachter in 1954 (1) found histamine, serotonin, and a potent smooth muscle stimulant which produced a characteristic delayed, slow contraction of the guinea-pig ileum in the presence of atropine and mepyramine and also after the muscle had been desensitized to serotonin. Activity was stable to heating at neutral pH but was rapidly destroyed in hot dilute NaOH or concentrated HCl. The similarity of the last smooth muscle stimulant in *Vespa vulgaris* to bradykinin was noted and its polypeptide nature was strongly indicated by Schachter and Thain (2), when it was shown that crystalline trypsin or chymotrypsin readily destroyed 50-70% of the activity. The factor was not precipitated by 60% ammonium sulfate, 5% trichloroacetic acid or 95% phenol but was insoluble in 95% ethanol, 95% acetone and anhydrous ether. In addition to producing the delayed slow contraction of the isolated guinea-pig ileum, crude venom also contracted the isolated rabbit jejunum and ileum, markedly lowered arterial blood pressure of the rabbit and to a lesser degree that of the cat. Differences between the active principle in wasp venom and known peptides led to its tentative designation as wasp kinin or simply kinin. Kinins are now regarded (3) as a group of peptides which have a variety of potent pharmacological properties in common. They are hypotensive, increase capillary permeability, contract most isolated smooth muscle preparations but relax the rat duodenum, produce pain and cause bronchoconstriction in the guinea-pig.

Wasp kinin has been purified from glacial acetic acid extracts of venom sacs (4). Addition of 15 volumes of anhydrous ether to the extract quantitatively precipitated the kinins which were washed with 95% ethanol, dissolved in water and lyophilized. The dry powder was stable at room temperature when stored in a desiccator. Using paper chromatography (butanol, acetic acid, water solvent system), purified wasp kinin could readily be distinguished from the known kinins as the former remained at the origin and the known kinins had an R_f of 0.35. Trypsin, which does not inactivate bradykinin and kallidin, destroyed 70-100% of wasp kinin activity. A pharmacological distinction was also noted in that venom kinin produced a secondary depression in the arterial blood pressure of the rabbit which was not consistently seen with bradykinin or kallidin. In other tests wasp kinin was 10-100 times more effective than histamine in increasing capillary permeability after intradermal injection and 10 times more effective than acetylcholine in evoking pain on a cutaneous blister base.

Chromatography of wasp kinin on columns of carboxylic acid exchange resin, amberlite XE-64 ($\equiv$ CG-50) also revealed differences (5). Of significance was the clear separation of the known kinins from the major wasp kinin which eluted later. This substance accounted for about 90% of the eluted activity. Two minor components (about 10% of the eluted activity) which eluted earlier than bradykinin and kallidin were also observed. Thus 3 kinins are present in *Vespa vulgaris* venom. Recovery of activity from the ion-exchange column was 30-50% for the wasp kinins and 15-30% for the known kinins. All three wasp kinin peaks had the same relative activity on the guinea-pig ileum and rat uterus and all were completely destroyed when incubated with chymotrypsin and much reduced inactivity by trypsin.

Hornet venom (*Vespa crabro*) like that of the wasp contains large amounts of histamine and serotonin but has in addition very high levels of acetylcholine and a single kinin with the characteristic kinin activity but which could be clearly distinguished from wasp kinin by chromatographic and enzymatic tests (6). Hornet kinin, like bradykinin, was found to be resistant to inactivation by trypsin. However, relative to bradykinin it was only about one tenth as active in contracting the guinea-pig ileum as in causing relaxation of the rat duodenum or lowering rabbit arterial blood pressure.

Unlike wasp or hornet venom, bee venom (*Apis mellifica*) contains little or no serotonin or kinin (2). It does, however, produce a delayed, slow contraction of the mepyramine-treated guinea-pig ileum. This activity is characterized by a rapid desensitization and may be due to an enzyme.

The most thorough study of the pharmacologically active substances in bee venom, spanning 16 years, has come from the University of Wurzburg (7). Histamine, hyaluronidase, phospholipase A and an active basic fraction were reported in earlier studies. Recent work on the further characterization of basic fraction has culminated in the isolation of two polypeptides, melittin and apamin (8).

Melittin forms micells in solution and this property may contribute to an understanding of its potency in lowering the surface tension of aqueous solutions and its powerful hemolytic action (9). The amino acid sequence of melittin, a

1 2 3 4 5 6 7 8 9 10

major component of bee venom is (X)-Gly-Ileu-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-

11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

-Thr-Gly-Leu-Pro-Ala-Leu-Ileu-Ser-Try-Ileu-Lys-Arg-Lys-Arg-Gln(NH_2)-Glu$(NH_2)_2$. Another minor melittin has also been characterized and differs from the major peptide by an unknown amino acid at the N-terminus before glycine and after

20 21 22 23 24 25 26

the identical sequence of the first 19 residues, Ileu-Ser-Arg-Lys-Lys-Arg-Glu(NH_2)-

27

-Glu$(NH_2)_2$ (10).

Apamin is present in much lower levels than melittin and is pharmacologically characterized by a long-lasting excitation of the central nervous system of mice. It has the following amino acid composition: Ala_3, Arg_2, Asp_1, $\frac{1}{2}Cys_4$, Glu_3, His_1, Leu_2, Lys_1, Pro_1, Thr_1 (8).

Unlike those of the bee, wasp and hornet peptides have pharmacological properties like the known kinins (Table I). These important actions as well as their potency make it of interest to determine their structures. The present report contains both a summary of earlier work (11, 12, 13) and the most recent findings which permit a proposal for the structure of the major kinin in *Polistes* venom.

STUDIES ON POLISTES

Materials and methods

Wasps

Three species of wasp *Polistes annularis* Linnaeus, *P. fuscatus* Lepeletier, and *P. exclamans* Viereck were delivered alive to the laboratory. These were mixed and when not immediately used they were stored at 3-5° for several days or up to two weeks at room temperature when fed canned or fresh fruit. Other foods such as cooked, strained meat or live silk works were rejected. Most of the wasps, up to 16,000 were immediately frozen at −10°. Their abdomens were dissected and the terminal 3 segments which contained the venom glands, sacs, ducts, and lancets were stored up to 6 months at −10°.

Extraction of venom and isolation of kinins

Several extraction procedures were compared in order to find the most convenient procedure for obtaining suitable quantities of material. An electric shock technique (14) was used to obtain pure venom which served as a standard. Extraction of venom apparatuses with glacial acetic acid as previously described (4) and homogenization of the dissected abdomen in 6% trichloroacetic acid were the other procedures employed. The essential steps in the latter procedure are: (a) homogenization of the dissected abdomens in 6% trichloroacetic acid, (b) removal of trichloroacetic acid from the extract with ether, (c) adsorption of the kinins on a preparative column of CM-Sephadex and elution with ammonium formate, (d) fractionation on Sephadex G-10, (e) fractionation on an analytical column of CM-Sephadex by gradient elution with ammonium formate, (f) repeat of step 5 using a flatter gradient.

Bioassays

Kinins were measured on the estrus rat uterus suspended in de Jalon's solution (12). Whenever serotonin may have been present, 1 μg/ml lysergic acid diethylamide was added to the bathing fluid. Antihistaminics were not added because the uterus was insensitive to the levels of histamine encountered. Bioassays using the guinea-pig ileum and rat duodenum were performed in the usual manner (15). Values were based on the responses to single and double doses of the unknown sample and standards. Rat blood pressure was recorded with a Statham Strain gauge and Sandborn Recorder.

Results

Essentially the same results, i.e., kinin activity equiactive to 1.2-1.5 μg bradykinin in the rat uterus assay, were obtained when venom was collected by electric shock, or extracted from apparatuses by glacial acetic acid, or extracted from abdomens with trichloroacetic acid. Because the latter procedure was more convenient, it was used to extract kinins from 6000 wasps. After the preparative CM-Sephadex step, kinin activity was obtained equiactive to 6.4 mg bradykinin. Chromatography on the analytical CM-Sephadex column resulted in the isolation

of 3 peaks of kinin activity. The same results were obtained when glacial acetic acid extracts of venom apparatuses were chromatographed on CM-cellulose (12). The first two peaks of activity, *Polistes* kinins 1 and 2, accounted for less than 10% of the total kinin activity and *Polistes* kinin 3 approximately 90%. Recovery of kinins from the columns was essentially quantitative.

In preliminary tests employing the estrus rat uterus, duodenum and blood pressure tests and the guinea-pig ileum, all three kinins showed activities similar to bradykinin and kallidin (Table 1); however, they could be distinguished from the known kinins in preliminary studies. For example when bradykinin was used as the standard *Polistes* kinin 3 was found to be about 7 times more active on the guinea-pig ileum than rat duodenum, 4 times more active in lowering rat blood pressure than on the ileum, 3 times more active on the ileum than on the uterus and 2 times more active on the uterus than duodenum. Thus relative to bradykinin *Polistes* kinin 3 was most active in lowering blood pressure, the order of activity being blood pressure > ileum > uterus > duodenum.

The structure of *Polistes* kinin 3 is currently under investigation. Incubation of the kinin with chymotrypsin destroyed biological activity but trypsin, pepsin or collagenase did not. The action of trypsin was particularly interesting because it cleaved the peptide into a fragment which was slightly more active on the uterus and which was readily isolated on a column of CM-Sephadex.

In a typical experiment, 1.0 mg of *Polistes* kinin 3 (recovered from CM-Sephadex and containing some salt and moisture) was incubated in 0.5 ml of 0.05 M ammonium bicarbonate pH 7.0 and 0.02 ml of 0.1% trypsin for 3 hours at 37°. This digestion caused an increase in bradykinin-like activity from 300 to 400 μg when tested on the rat uterus. When analyzed on a CM-Sephadex column no activity was recovered where *Polistes* kinin 3 is normally found. Instead activity equivalent to 390 μg bradykinin was found as a single peak much earlier than the position of *Polistes* kinin 3. Amino acid analysis of the new active peptide revealed that it had the same composition as bradykinin but, in addition, also contained glycine. Comparison by TLC of the dansyl derivative of the active tryptic peptide and synthetic glycylbradykinin showed they were indistinguishable (13). Dansyl glycine was identified in an acid hydrolysate of the peptide derivative showing that glycine was at the N-terminus. Additional evidence for the identity of the active tryptic fragment of *Polistes* kinin 3 as glycylbradykinin was the identical potencies on the rat uterus of the natural and synthetic peptides.

Acid hydrolysates of the wasp kinin showed that in addition to the amino acids in glycylbradykinin there are 3 residues of lysine and 1 each of glutamic acid, threonine, aspartic acid, leucine and arginine. Experiments performed in our laboratory by Dr. T. Nakajima have indicated that the N-terminus is blocked, as the peptide did not react with leucinaminopeptidase nor in the Edman procedure. Furthermore only ε-dansyl-lysine was observed in hydrolysates of the dansylated peptide. Upon treatment with trypsin the following were observed: free lysine, leucylarginine, glycylbradykinin and an N-terminal blocked tetrapeptide which after acid hydrolysis was found to contain glutamic acid, threonine, aspartic acid and lysine.

Reaction of the wasp kinin with carboxypeptidase B yielded only free arginine which was also observed after treatment with chymotrypsin. Horse urinary kallikrein, like trypsin, also split glycylbradykinin from the wasp kinin but only

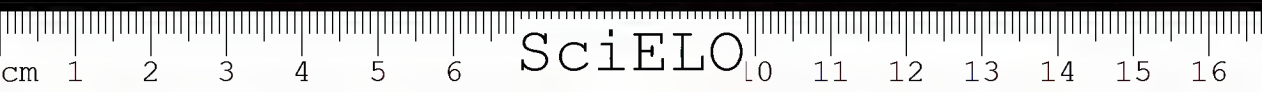
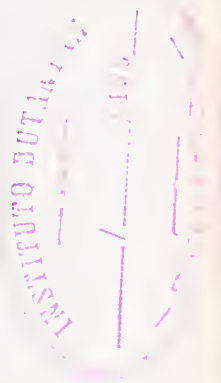



TABLE I — COMPARISON OF KININS

KININ	Smooth muscles [2]	Rat duodenum	Capillary permeability [3]	Blood pressure [4]	Pain production [5]	Chymotrypsin	Trypsin
Bradykinin	Contract	Relax	Increase	Hypotensive	Yes	Inactivates	No inactivates
Kallidin	"	"	"	"	"	"	"
Vespa vulgaris [1]	"	"	"	"	"	"	Inactivates
Vespa crabro	"	"	"	"	Not tested	"	No inactivates
Polistes [1]	"	"	"	"	"	"	" [6]

1. *Vespa vulgaris* and *Polistes* venoms contain 3 kinins which, when tested, were found to have similar pharmacological actions.
2. Guinea-pig ileum, rat uterus, rabbit jejunum and ileum. *Polistes* kinins were not tested with the rabbit preparations.
3. Guinea-pig skin.
4. Rat, rabbit and cat. *Polistes* kinins were tested only on the rat.
5. Man.
6. Although trypsin did not cause inactivation it did hydrolyze *Polistes* kinin 3 and an active fragment was isolated and identified as glycylbradykinin. See text.

one additional peptide was found which upon acid hydrolysis yielded 3 residues of lysine and one each of glutamic acid, threonine, aspartic acid, leucine and arginine. These results are in agreement with present knowledge of the specificity of horse urinary kallikrein which is known to be an endopeptidase which splits only arginyl bonds (16). If the wasp peptide is first dansylated, in which case all the ε-amino groups of lysine react, then trypsin and horse urinary kallikrein gave the same products, i.e., glycylbradykinin and a second peptide containing all the remaining amino acids, plus ε-dansyl lysine. The data up to this point indicate the following structure: (Glu, Thr, Asp, Lys_2) Lys-Leu-Arg-Gly-bradykinin. Enzymatic digestion of *Polistes* kinin 3 with a combination of Pronase, carboxypeptidases A and B, trypsin, chymotrypsin C and prolidase yielded asparagine, pyroglutamic acid and pyroglutamylthreonine. Aspartic or glutamic acid was not observed and threonine was lowered by an amount expected from the pyroglutamylthreonine found. Other data obtained with trypsin and chymotrypsin C indicate that the 3 lysine residues are sequential. Hence the following tentative structure for *Polistes* kinin 3 is: Pyr-Thr-Asn-Lys-Lys-Lys-Leu-Arg-Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg.

Identification of the bradykinin structure in wasp venom, together with an earlier report on its presence in amphibian skin (17) indicate the widespread occurrence of the kinin in nature and support accumulating evidence for the significance of kinins in man. Certainly, their presence in such a highly specialized fluid as venom which also contains well-known pharmacologically active substances, including histamine, serotonin, acetylcholine, phospholypase A, hyaluronidase, suggests that kinins and particularly the bradykinin molecule are uniquely active substances of physiological importance.

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48. PURIFICATION DES NEUROTOXINES DU SCORPION *ANDROCTONUS AUSTRALIS*

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Des recherches antérieures (1-3) on montré que les venins de deux espèces de scorpions Nord-Africains contenaient chacun deux neurotoxines dont la purification a été réalisée par rétention réversible sur Sephadex G-25 et par chromatographie d'échange d'ions sur Amberlite IRC-50. Les protéines basiques obtenues étaient homogènes en ultracentrifugation, en électrophorèse de zone sur gel d'amidon et en chromatographie d'équilibre sur Amberlite IRC-50. Un poids moléculaire de 11.000 à 18.000 avait été obtenu par ultracentrifugation. Des travaux ultérieurs ont montré que le traitement des toxines à des pH éloignés de la neutralité conduisait à leur dissociation en monomères.

Une nouvelle méthode de purification a été mise au point, permettant d'obtenir les toxines monomères avec un rendement élevé. Le matériel de départ était le venin brut provenant d'animaux collectés à Tozeur (Tunisie), recueilli par stimulation électrique et desséché sous vide. La purification a comporté essentiellement, une extraction par l'eau, une filtration sur Sephadex G-50, suivie de deux chromatographies successives sur Amberlite IRC-50 à pH 6,70 et sur DEAE-Sephadex A-50 à pH 8,50. Une dernière chromatographie d'équilibre sur Amberlite IRC-50 à pH 6,30 pour la toxine I et à pH 6,70 pour la toxine II a conduit à l'obtention des toxines pures. Comme dans la méthode précédente, les différentes séparations chromatographiques ont été réalisées en utilisant des tampons acétate d'ammonium, dont le sel peut être complètement éliminé par une *double* lyophilisation. Il a été ainsi possible d'éviter des opérations de dialyse-concentration, source de pertes appréciables de toxines. Un exposé détaillé de la procédure de purification pourra être trouvé dans un article à paraître prochainement (4). Le tableau I résume les étapes de la purification. 52 mg de toxine I soit 1,6% et 83 mg de toxine II soit 2.5% du venin brut de départ ont été obtenus. La toxicité retrouvée dans les toxines pures correspond à 65% de celle du venin brut. Des résultats parfaitement reproductibles ont été obtenus au cours d'opérations répétées de purification portant sur 20 g de venin.

La DL_{50} des toxines pures déterminée sur la souris de 20 g en présence d'albumine et par voie intraveineuse est de 19 μg/kg (toxine I) et de 10 μg/kg (toxine II). Les toxines pures sont donc 10 et 19 fois plus neurotoxiques que le venin brut. La composition en acides aminés des toxines pures est rapportée dans le tableau II. On notera l'absence de méthionine dans les deux toxines et celle d'acide glutamique dans la toxine I. L'absence de cystéine a été constatée par titrage par le p-chloromercuribenzoate (9). Ce résultat a été confirmé par

TABLEAU I — PURIFICATION DES NEUROTOXINES DU VENIN *D'ANDROCTONUS AUSTRALIS*

ETAPES			DL_{50} (nombre)*		Toxicité specifique**	Rendement en toxicité (%)***	
Venin recueilli par stimulation électrique (3,224 g)			844.383		283	100,0	
Extraction par l'eau et dialyse (48 heures)			837.628		310	99,2	
Filtration sur Sephadex G-50			775.988		622	91,9	
Chromatographie d'équilibre sur Amberlite CG-50 à pH 6,70 (tampon $AcNH_4$ 0,2 M)	Fraction toxique I		157.900	599.512	1.240	18,7	71,0
	Fraction toxique II		441.612		1.675	52,3	
Chromatographie d'équilibre sur DEAE-Sephadex à pH 8,50 (tampon $AcNH_4$ 0,1 M)	Fraction toxique I		146.923	569.959	1.602	17,4	67,5
	Fraction toxique II		423.036		1.800	50,1	
Chromatographie d'équilibre sur Amberlite CG-50 (tampon $AcNH_4$ 0,2 M)	à pH 6,30	Toxine I	137.634	553.915	1.680	16,3	65,6
	à pH 6,70	Toxine II	416.281		2.100	49,3	

* Déterminée selon BEHRENS et KARBER (5) par injection intraveineuse à la souris Swiss mâle de 20 g de solutions toxiques supplémentées avec de la sérumalbumine humaine 1 × cryst. (2 mg/ml).

** DL_{50}/unité DO à 280 mμ.

*** Par rapport à l'étape initiale.

$AcNH_4$ = acétate d'ammonium.

l'hydrolyse (HCl 6N à 110° pendant 20 et 70 h) des toxines alkylées par l'acide monoiodoacétique qui ont fourni 7,40 (toxine I) et 7,52 résidus (toxine II) de demi-cystine et aucun résidu de S-carboxyméthylcystéine.

Le poids moléculaire déterminé par équilibre de sédimentation selon Sved-
monoiodoacétique qui ont fourni 7,40 (toxine I) et 7,52 résidus (toxine II) de
minations ont été réalisées dans une ultracentrifugeuse Spinco E à 15.220 et 13.410 rev./min (toxines I et II) pendant 88.5 et 116 h à 20°. Les toxines étaient dissoutes dans l'acétate d'ammonium 0,20 *M* pH 6,90 à une concentration de 0,8%. Les valeurs obtenues sont très proches du poids moléculaire minimum calculé d'après la composition en acides aminés (6.822 et 7.249 pour I et II).

Dans l'acide acétique 0,5 N, les toxines I et II présentent des maximum d'absorption à 275 et à 276 mμ respectivement. Pour ces longueurs d'onde les coefficients d'extinction moléculaire sont $10{,}71 \times 10^3$ (toxine I) et $18{,}08 \times 10^3$ (toxine II).

TABLEAU II — COMPOSITION EN ACIDES AMINÉS DES NEUROTOXINES D'*ANDROCTONUS AUSTRALIS*

1 à 2 mg de toxine pure ont été hydrolysés pendant 20 et 70 h dans HCl 6N à 110° selon MOORE et STEIN (7) et analysés par chromatographie sur colonne selon PIEZ et MORRIS (8) avec un Autoanalyzer Technicon. Chaque valeur représente la moyenne de deux analyses pour chacun des temps d'hydrolyse. Les valeurs de la sérine, de la thréonine et de la tyrosine ont été calculées par extrapolation au temps d'hydrolyse 0.

Acide aminé	Toxine I (rapport molaire *)		Toxine II (rapport molaire *)	
A. aspartique	9,04	(9)	8,13	(8)
Thréonine	2,00	(2)	3,07	(3)
Sérine	5,76	(6)	2,12	(2)
A. glutamique	0,0	(0)	4,13	(4)
Proline	5,96	(6)	2,86	(3)
Glycine	6,04	(6)	7,02	(7)
Alanine	1,06	(1)	3,12	(3)
Cystine (1/2)	7,55	(8)	7,92	(8)
Valine	4,29**	(4)	4,08	(4)
Méthionine	0,0	(0)	0,0	(0)
Isoleucine	2,46**	(3)	0,98	(1)
Leucine	4,01**	(4)	1,75	(2)
Tyrosine	2,83	(3)	7,04	(7)
Phénylalanine	1,01	(1)	0,99	(1)
Lysine	5,87	(6)	5,00	(5)
Histidine	0,99	(1)	1,96	(2)
Arginine	2,03	(2)	2,99	(3)
NH_3 amidé		(6)		(9)
Tryptophane ***		(1)		(1)
Total		63		64
Poids moléculaire minimum	6.822		7.249	

* En prenant phénylalanine = 1,0. Entre parenthèses l'entier le plus proche.
** Valeur obtenue après 200 h d'hydrolyse.
*** Détermination spectrophotométrique selon BEAVEN et HOLIDAY (6).

Les acides aminés N-terminaux ont été déterminés par dinitrophénylation selon Fraenkel-Conrat *et al.* (10). Pour les deux toxines, une seule tache a été observée sur les chromatogrammes. Elle correspondait à la di-DNP-lysine dans plusieurs solvants chromatographiques et, après élution du papier, elle en présentait le spectre caractéristique. La recherche des acides aminés C-terminaux a été réalisée par hydrazinolyse selon Akabori *et al.* (11). Après analyse chromatographique sur colonne, on a trouvé la thréonine pour la toxine I et le glycocolle pour la toxine II. *Aucun* autre acid aminé n'a été décelé.

Les preuves de l'homogénéité des toxines sont les suivantes: 1) par électrophorèse en gel de polyacrylamide à pH 3,6 et en présence d'urée 8M (12), chacune des toxines migre sous forme d'une bande unique. Il en est de même en gel d'amidon à pH 8,6 où les bandes des toxines se déplacent du côté cathodique, indiquant leur caractère fortement électropositif. 2) le fait que chacune des toxines sédimente dans l'ultracentrifugeuse analytique en donnant une frontière unique et symétrique ne peut être considéré que comme une présomption d'homogénéité en raison de l'existence dans le venin brut d'autres protéines non toxiques de faible poids moléculaire. 3) après rechromatographie d'équilibre sur Amberlite IRC-50 les fractions constituant le pic toxique symétrique obtenu ont une toxicité spécifique constante et maximum. 4) l'absence de méthionine dans les deux toxines et d'acide glutamique dans la toxine I constitue un critère très sensible de la pureté des fractions obtenues. 5) un seul acide aminé N- ou C-terminal a été mis en évidence pour chacune des toxines.

Un certain nombre d'arguments milite en outre en faveur de ce que chaque toxine est constituée par une chaîne polypeptidique *unique*:

1) Après réduction complète des ponts disulfures et alkylation (acide monoiodoacétique ou iodacétamide), on devrait s'attendre, si les toxines étaient constituées par deux ou plusieurs chaînes polypeptidiques, à ce qu'elles pénètrent dans le Biogel P_6 (qui exclut les molécules d'un poids égal ou supérieur à 4.600) et qu'elles soient plus retardées que les toxines natives sur Sephadex G-50. Tel n'est pas le cas. Les toxines réduites et carboxyméthylées sont exclues du Biogel P_6 et moins retardées que les toxines natives sur Sephadex G-50. De plus elles dialysent beaucoup moins vite que les toxines natives à travers la même membrane semi-perméable.

2) Un seul acide aminé N-terminal a été trouvé. Bien que la présence éventuelle d'acides aminés terminaux N-acylés n'ait pas été recherchée, leur existence est rendue peu vraisemblable du fait qu'un seul acide aminé C-terminal a été trouvé dans chacune des deux toxines dans des proportions n'excédant pas un résidu par molécule.

L'ensemble des résultats obtenus permet d'assigner aux neurotoxines d'*Androctonus australis* les formules linéaires suivantes:

Toxine I: H-Lys-(Asp_9, Thr_1, Ser_6, Pro_6, Gly_6, Ala_1, $CyS\text{-}SCy_4$, Val_4, $Ileu_3$, Leu_4, Tyr_3, Phe_1, Lys_5, His_1, Arg_2, Try_1)-Thr-OH.

Toxine II: H-Lys-(Asp_8, Thr_3, Ser_2, Glu_1, Pro_3, Gly_6, Ala_3, $CyS\text{-}SCy_4$, Val_4, $Ileu_1$, Leu_2, Tyr_7, Phe_1, Lys_4, His_2, Arg_3, Try_1)-Gly-OH.

Ces formules, qui n'impliquent pas de séquence entre les acides aminés inclus dans la parenthèse, montrent certaines analogies entre les deux toxines: 1)

le nombre total d'acides aminés (63 et 64). 2) l'absence de méthionine et la présence de quatre ponts disulfures. 3) la richesse en acides aminés aromatiques (5 et 9 résidus pour I et II). 4) le même résidu de lysine N-terminal.

Des caractéristiques voisines ont été mises en évidence dans la toxine I de *Buthus occitanus* (2), compte tenu de ce que l'on sait maintenant que les analyses précédemment publiées portaient sur le dimère de la protéine.

La symptomatologie de l'envenimement produit par l'injection des toxines est identique pour chacune d'elles et pour le venin brut desséché ou non. Etant donné l'absence pratiquement totale d'activités enzymatiques dans le venin d'*Androctonus australis*, on peut conclure que la symptomatologie de l'envenimement est directement et seulement liée à la présence des neurotoxines dans le venin.

Malgré leur différence de composition en acides aminés, les analogies de structure existant entre les deux neurotoxines étudiées et l'identité de leur action pharmacologique permettent d'envisager qu'un motif structural unique dans chacune des protéines pourrait être responsable de leur activité neurotoxique.

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Discussion

A. do Amaral: "Est-ce que le pouvoir antitoxinogénique de ces deux toxines a déjà été essayé comparativement?"

S. Lissitzky: "Non".

D. Mebs: "Have you found a direct relation between the toxicity and the reduction of the SS-bonds of your neurotoxin preparation?"

S. Lissitzky: "The fully reduced and carboxymethylated toxins have lost their toxicity."

E. G. Mendes: "Les deux toxines ont-elles le pouvoir de modifier l'activité cholinestérasique?"

S. Lissitzky: "Cet effet éventuel n'a pas été essayé."

A. Shulov: "Our group has an impression that there is a difference in constituents whether the venom is lyophilised, fresh or from direct bites. We shall be glad to help your group in the preparation of anti-serum against *Androctonus australis* if you would like to try to elucidate this difference, according to the method which led to excellent results your group achieved."

S. Lissitzky: "I thank you for your suggestion, Dr. Shulov, and I should be glad to get in touch with you."

N. Sarkar: "It is unusual that a polypeptide of such molecular weight as the scorpion venom is so easily denatured. Do you know which are the structural properties responsible for this behaviour of the polypeptide?"

S. Lissitzki: "I think we should wait until more information on the structure of the toxins is available."

C. Y. Lee: "Is there any study on the action of your purified neurotoxins on the neuromuscular transmission?"

S. Lissitzki: "We have not done such studies."

J. M. Gonçalves: "Etant donné que vous avez, par hydrazinolise, identifié la glycine comme C-terminal de la toxine II, avez-vous trouvé une résistence de la toxine à l'action de la carboxipeptidase du pancreas?"

S. Lissitzki: "Ces essais sont en cours de réalisation."

49. CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITY OF *TITYUS* VENOM

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The two most prevalent species of scorpions in Brazil are the yellow scorpion *T. serrulatus* and the black scorpion *T. bahiensis*. Their venoms produce on mammals similar symptomatologies but the degree of toxicity is not the same (1; 2). Attempts to fractionate these venoms, obtained by electrical stimulation, led Diniz and Gonçalves (3, 4) to demonstrate that they are mixtures of proteins, mostly of basic nature; the main method used was paper and starch zone electrophoresis. The following components were found: a) toxic b) smooth muscle stimulating c) increasing capillary permeability and d) hyaluronidase; *a* and *b* were inactivated by proteolytic enzymes. From several enzymes assayed, only *d* showed measurable activity.

Recently Gomez and Diniz submitted *T. serrulatus* venom to a fractionation procedure using a combination of precipitation and column chromatography on Sephadex G-25 and CM-cellulose; they have confirmed the protein complexity of the venom and, in addition, isolated a highly purified toxic component which migrated as a single component on cellulose acetate paper electrophoresis (Fig. 1).

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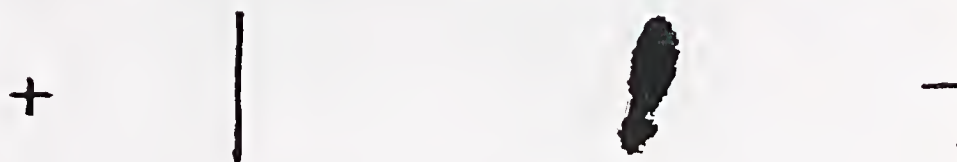
Tityus serrulatus toxin
Cellulose acetate paper electrophoresis
Acetate buffer pH 5.5, μ 0.05, 7 hours

Fig. 1 — Cellulose acetate paper electrophoresis of the toxic component obtained after chromatography of the T_2 component in a CM cellulose column. Acetate buffer pH 5.5 μ = 0.05. A single component was obtained.

Mechanism of action of Tityus venom

As was pointed out in previous paragraphs, the venomous secretions are complex mixtures. When the whole venom or extracts of the venom apparatus are used to study the venom mechanism of action, difficulties to interpret results

arise. In the case of scorpion venoms, use has been made in general either of telsons extracts or of whole venom obtained by electrical or manual stimulation. In most cases, following injections into whole animals, the effects observed on blood pressure, heart rate, respiration, glandular secretions, etc., are used to attempt an understanding of the mechanism of action. In spite of the difficulties involved several authors as Houssay (5), Magalhães (1), Carvalho (6), Ramos and Corrado (7), Freire-Maia and Ferreira (8) have reached the conclusion that the *Tityus* venom contains neurotoxic substances.

The study of the effects of *Tityus* venom on isolated organs disclosed some effects that point to the parasympathetic nerves as the mediators of the toxic action (3, 9). *Tityus* venom contracts the smooth muscle of the guinea-pig ileum. This effect is inhibited by atropine and potentiated by eserine; ganglion blocking agents such as hexamethonium have no effect and morphine partially antagonizes the activity of the venom on this preparation. These results (Fig. 2) led to the supposition that *Tityus* venom contained at least
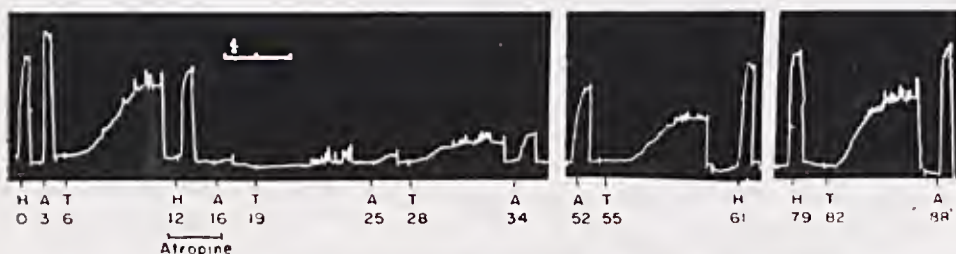
Fig. 2 — Guinea-pig ileum preparation suspended in 10 ml Tyrode solution. 0.4 μg of atropine sulphate left 2 minutes in contact with the preparation inhibits the action of subsequent additions of 5 μg of toxin preparation (T) or 0.08 μg of acetylcholine chloride (A); 0.04 μg of histamine (H) is not affected. The recovery time is about the same for the toxin and acetylcholine. (According to Diniz and Valeri, 1959).

one component acting on parasympathetic postganglionic fibers (9). This effect might be either a direct one or mediated through an autopharmacological substance present in the nervous endings of guinea-pig ileum plexuses. Recently, in collaboration with Torres (10), we incubated fragments of guinea-pig ileum with *Tityus* venom and tested the release of acetylcholine according to Paton (11); in the flasks containing venom, a smooth muscle contracting substance, with pharmacological and some chemical properties similar to acetylcholine, accumulated. We concluded that the contracting effect of the venom on the guinea-pig ileum is indirect and mediated through acetylcholine. Since the venom does not inhibit cholinesterase (4) the release of acetylcholine must be due to an effect of the venom in some other place of the metabolic pathway of this substance. Many symptoms of the scorpion venom intoxication could be interpreted by admitting the release of acetylcholine in the nervous structures.

These results were, however, in apparent discordance with the findings of Ramos and Corrado (7); Freire-Maia and Ferreira (8) who found that in some conditions the venom produced hypertension, tachycardia, hyperglycemia, relaxed the atropinized rabbit duodenum and produced other symptoms suggestive of sympathetic mediation. We decided thus to investigate the action of the venom using the perfused guinea-pig heart which offered the possibility to analyse in the same structure effects mediated by acetylcholine or adrenaline, without the interference of the complex structures of the whole animal.
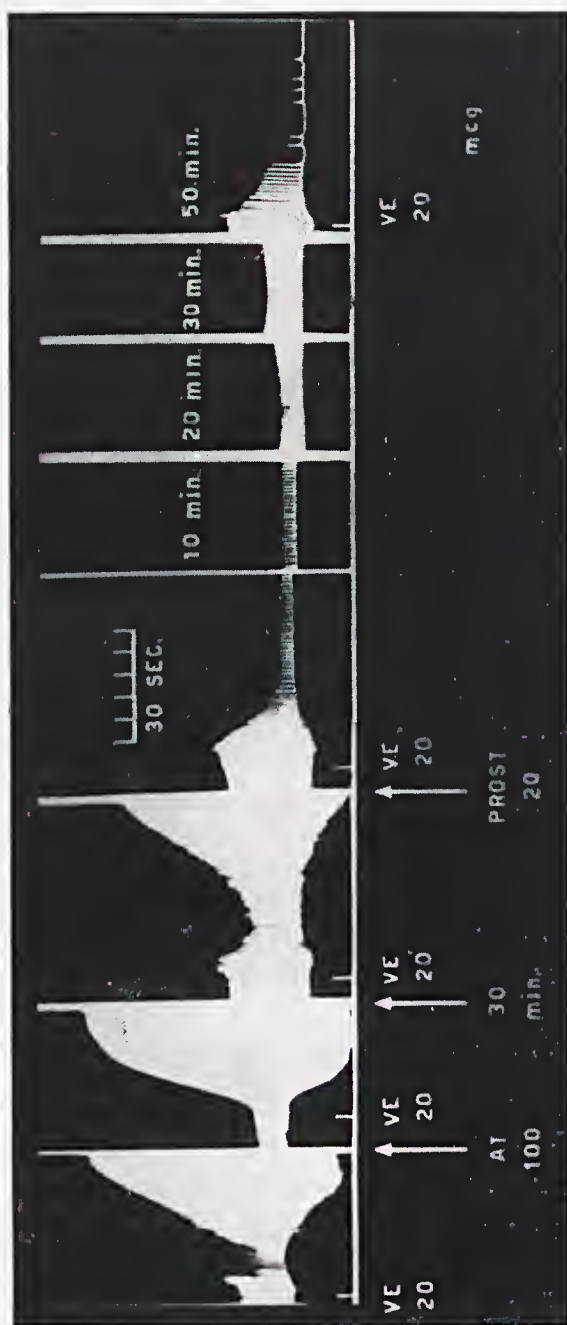
Fig. 3 — Effect of the perfusion of 20 μg venom on the isolated guinea-pig heart. Note the negative, followed by positive, chronotropic and inotropic effect. Atropine abolishes and prostigmine potentiates the negative effects.

We were able to show that 20 μg of venom perfused through the heart produced first bradycardia simultaneously with a reduction of strenght of heart beats. These experiments suggested a participation of cholinergic, simultaneously or followed by adrenergic mechanisms in the action of the venom (Fig. 3). This hypothesis was confirmed by use of drugs which interfere with both mechanisms. The negative chronotropic and inotropic effects were blocked by atropine and hemicholinium and potentiated by prostigmine. Hexamethonium had no effect; *beta* sympatholitics, as Inderal, inhibited the positive effects. Previous treatment of the guinea-pig with reserpine, a substance known to deplete catecholamines, prevented the positive inotropic and chronotropic effects, allowing however the negative effects to show.

The important point now is to decide if these effects are produced by different components or by the same substance. We cannot decide at the moment, but the toxin obtained in the various steps of the purification procedure, presented both effects on the heart. Based on these results, we can speculate about the site of action of the venom. A hypothesis which satisfies the results obtained in our experiments is that the venom is a postganglionic fiber neurotoxin. If we admit with Burn and Rand that even noradrenaline is liberated by a previous release of acetylcholine in the adrenergic nerves, only one toxic substance, that may act always by releasing acetylcholine from the autonomous nervous system, can explain all the effects hitherto observed in the isolated organs.

We may conclude these remarks by pointing out the interesting possibilities offered by the study of interaction of *Tityus* toxin with the structures of the nervous system.

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VII

FARMACOLOGIA

PHARMACOLOGY

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50. PHARMACOLOGY OF VENOMS — INTRODUCTORY REMARKS

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The present Introductory Remarks on the Pharmacology of Venoms should cover a large area treated in this Symposium, since most of the subjects which are going to be discussed here have a bearing on the Pharmacology of Venoms. Especially all papers on the biochemical aspects of the action of venoms are of the utmost importance to understand their pharmacological actions.

Obviously, I have to limit my talk to a few personal recollections about our past and present work involving animal venoms.

In the beginning, I was mainly interested in the mechanism of anaphylaxis and related phenomena, from the point of view of a release of histamine. Consequently, I became chiefly interested in the work by Feldberg and Kellaway (1937-38), in Australia, about the release of histamine by the Australian and Indian cobra, the *Denisonia superba* and the *Naja naja*. Both venoms, when perfused through the guinea-pig lung, produced a sharp release of histamine, leading almost to exhaustion of the stock of this amine in that organ of the guinea-pig. However, other experiments by the Australian workers, on the circulatory effects of the venoms, as well as on the effects upon the isolated smooth muscle of the guinea-pig ileum, were consistent with the idea that besides histamine, other endogenously released principles might participate in such envenomations. An important outcome of these experiments was the conclusion that, under certain conditions, a slow-reacting substance (SRS) was released and could explain some of the features of the shocks by animal venoms.

These experiments showing the release of histamine and of a slow-reacting substance, by snake venoms, established an obvious parallelism between the symptoms of envenomation and those of anaphylaxis, as had been described in the dog, by Richet and Portier (1902), in the guinea-pig, by Theobald Smith (1906) and in the rabbit, by Arthus and Breton (1903-5).

This analogy was the most striking having in view the way by which anaphylaxis was discovered by Richet and Portier, in 1902. It is well known that the French physiologists were studying the venom of the sea anemona, of the genus *Physalia* and *Actinia*, using glycerinated extracts of their tentacles. When injected with a lethal dose of these extracts the animals showed a picture similar to that they could have under the anaphylactic crisis: diarrhea, abdominal cramps, fall in blood pressure, coma and death.

By section, the animals showed profuse hemorrhages and stagnation of blood in the portal region.

The new phenomenon, observed by Richet and Portier and to which they gave the name of *Anaphylaxis*, was that a similar picture could be obtained with a small fraction of the lethal dose, if such a small amount of the extracts was given 15 to 20 days after the animal had been submitted to a previous injection of a non-lethal dose of the extracts. Therefore, they have concluded that instead of becoming protected (immunized) by a previous administration of the toxin, the animals presented an increased sensitivity to it. To denote this absence of protection or *phylaxis*, Richet and Portier coined the name which became famous of *Anaphylaxis*.

This story is well known to all those who have worked in anaphylaxis, but the point I wish to raise, is the implication contained in that name of Anaphylaxis, that the primary agent should be the toxic material and that the symptoms arising at the second injection were only exacerbation of the primary toxic effects of the utilized material. What Richet and Portier tried to imply with the name of anaphylaxis was the reduction of *protection* to the toxic effects of the glycerinated extracts of *Physalia* or *Actinia* tentacles. Later on, the name was widely utilized to indicate the development of toxic effects by materials, such as serum, ovalbumin and so forth, which are primarily non-toxic and become so after the repetition of the treatment.

In 1904, Arthus discovered sero-anaphylaxis, i.e., the development of toxic effects in the rabbit by repeated injections of horse serum, and in 1906, the immunologists with Theobald Smith, described in guinea-pigs the anaphylactic reaction to the reinjection of sera to which the animal is normally insensitive. We know what great success had this name of Anaphylaxis to indicate a great deal of pathological phenomena in animals and in humans, where von Pirquet and Schick described serum disease and allergy.

But, why should Richet be fooled in his experiments to the point to think that what he was observing at the second injection was in reality the *absence of protection* towards the primary toxic effects of his material extracted from the tentacles of the *Actiniae?* It is easy now to answer such a question. In fact, the symptomatology produced by many venoms from animal origin, resembles or mimics the effects that we know to be typical of Anaphylaxis. In other words, what he took as an intensification of the primary effects of his toxin, that he used to call *actinocongestin*, were actually the similar symptoms produced by Anaphylaxis, which occur as a consequence of an endogenous intoxication, by release of active substances, among which histamine is certainly one of the most important, at least in dogs and guinea-pigs. But, of course, the actinocongestin when acting as a toxic material releases histamine and/or other mediators directly from their endogenous stores, though when acting in the sensitized animal to produce anaphylaxis, the symptomatology develops through an entirely different mechanism, namely by combination with antibodies formed after the first injection. It is this profound analogy between envenomation by animal poisons and the symptomatology of Anaphylaxis that was so nicely explored by Arthus in his book "De l'Anaphylaxie à l'Immunité", published in 1921, in Paris.

But, in the meantime, the important contribution by Sir Henry Dale, in England, studying the effects of histamine and postulating its participation in the mechanism of Anaphylaxis, should be considered.

In 1919, Dale proposed the name of *Autopharmacology* to denote this class of phenomena developing in the animal body by the release of endogenous active materials, especially histamine.

And then, the excellent work which followed, by Feldberg and Kellaway (1937-38) and others to show the importance of the release of histamine and slow-reacting substances (SRS) in the mechanism of production of shocks by snake venoms.

Another kind of shock in which histamine appears to participate to a very important extent is the shock produced by *Ascaris*, in guinea-pigs and dogs. This kind of shock was studied by myself, with Graña, Porto and Andrade (in 1945-46), from the point of view of a release of histamine. It is enough to take a portion of a single worm, to macerate it in saline and inject into a 10 kg dog to produce an extremely severe shock, resembling in all details anaphylaxis in this species. For that reason we have called this shock an *Anaphylaxis-like* reaction, instead of using the common expression of *Anaphylactoid*. By that time, I thought that the phenomenon could be explained as *true* anaphylaxis, assuming that the dogs were probably sensitized to the *Ascaris* material. In agreement with this point of view, Beraldo and his colleagues, in 1961, have presented evidences that the effect of *Ascaris* in the guinea-pig appears to depend upon a real state of sensitization, probably as a consequence of infestation by **NEMATODA** parasites, which are common in such animals.

I cannot dwell any longer upon this interesting aspect of the phenomenon, that once more established so close connections between Anaphylaxis and the envenomation by animal venoms.

In the preceding discussion we have mentioned mainly histamine as the principle released by snake venoms and *Ascaris* extracts.

It was, indeed, with this idea in mind that, in 1948, we started doing experiments to decide whether the venom of *Bothrops jararaca* produces its symptomatology in dogs, by releasing histamine from the liver. We were doing, with Beraldo, liver perfusions and have assayed the venom, brought to us by Rosenfeld, to see whether it releases histamine from dog's liver. Since we had shown, in our previous experiments with *Ascaris* extracts, that the blood was important for the release of histamine, we perfused the liver with defibrinated blood to which the venom was added at the moment of the perfusion. The output was not histamine, but bradykinin and it became clear that the venom released this material from the globulin fraction and that besides the venom, also trypsin would release bradykinin. But, what was most important, the liver itself had nothing to do with the release of the new substance, and if we added the venom directly to the defibrinated blood or to the pseudo-globulin prepared from it, the same activity or even more, was released and could be demonstrated upon the isolated guinea-pig ileum, made insensitive to the venom by repeated previous additions of the same.

But here, again, we had a strong analogy between the action of trypsin, which was found to produce very strong anaphylactoid symptoms when given to dogs and rabbits, and the envenomation by snake venoms. Those who are present to this section of Pharmacology know very well, and some better than I, the conditions in which bradykinin is released by venoms and toxins. I will use the few minutes still left to discuss some of the pharmacological actions of bradykinin: as a vaso-dilator agent not only upon the systemic but also upon the coronary circulation, as a powerful stimulating substance upon the smooth muscle of the intestinal tract in certain animal species and the isolated uterus of many species tested, as the most powerful agent to produce increased capil-

lary (vascular) permeability and as a mediator of pain. Recently, I have assembled a "Bibliography on Bradykinin" which, though incomplete, covers around 900 items.

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51. LIBERATION OF PHARMACOLOGICALLY ACTIVE SUBSTANCES FROM MAST CELLS BY ANIMAL VENOMS

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In many instances, reactions following contact or parenteral administration of animal venoms are indistinguishable from anaphylactoid reactions. For the first time the analogy between antigen administration in a sensitized animal and the action of a snake venom (*Crotalus adamanteus*) was clearly demonstrated by Arthus (1).

It is now generally accepted that during anaphylaxis several biologically active substances are liberated and activated from precursors (histamine, 5-hydroxytryptamine, heparine, slow reacting substances, kinins) (2, 3, 4).

On the basis of the similarity between the effects of snake venoms and histamine (5, 6, 7), the liberation of histamine from perfused tissues by animal venoms was investigated by Feldberg and Kellaway (8). The release of histamine was first attributed to the phospholipase activity of the venom and to the lysophosphatides formed (9). Rocha e Silva and coworkers (10, 11) demonstrated the liberation of histamine from isolated organs following perfusion with proteolytic enzymes and snake venoms. Besides histamine, slow reacting substance appears in the perfusion fluid of isolated lung under the influence of snake and bee venoms (12). 5-hydroxytryptamine is liberated by animal venoms from platelets (13). Bradykinin can be formed from the globulin fraction of normal plasma (bradykininogen) under the influence of snake venoms or trypsin (14, 15).

In anaphylactic and anaphylactoid reactions (administration of animal venoms) identical mediators are formed and liberated (16). In both types of reactions, tissue mast cells play an important role. Certain chemical substances as well as enzymes produce anaphylactoid reactions by degranulating mast cells and by liberating biologically active substances; concomitantly kinins are activated from precursors.

In Table I, a summary is given of direct and indirect mediators of anaphylactic shock, which are found in animal venoms.

Histamine has been identified in the venoms of **HYMENOPTERA** (17. 18), **DIPTERA** (19), **LEPIDOPTERA** (20), **HEMIPTERA** (21), *Scolopendra* (22) and **OCTOPODA** (23).

5-Hydroxytryptamine is present in the venoms of **ACTINARIA** (24, 25), **GASTROPODA** (26), **CEPHALOPODA** (27, 28) **HYMENOPTERA** (29), **ARACHNIDA** (30, 31) and in skin secretions of **AMPHIBIA** (32, 33, 34).
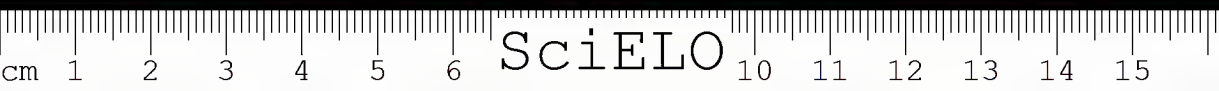
TABLE I — ANAPHYLACTOID SUBSTANCES IN ANIMAL VENOMS

DIRECT EFFECTORS:
Histamine
5-hydroxytryptamine (5-HT, serotonin)
kinins
INDIRECT EFFECTORS:
(Histamine, 5-HT, kinins)
Enzymes (proteases, phospholipase, esterases, hyaluronidase)
Kinin-producing fraction
Anaphylatoxin-producing fraction
High molecular mast cell depletors of unknown structure
Low molecular mast cell depletors of known structure
Surface active agents

A characteristic *kinin* was found in wasp venom (26, 35, 36, 37). In the venom of *Bothrops jararaca* a bradykinin potentiating factor has been found (38).

It should be mentioned that histamine (39, 40), 5-hydroxytryptamine (41, 39, 40) and kinins (42) exert a mast-cell depleting effect; for this reason. these substances are considered to be indirect effectors of anaphylactoid shock, too.

Several animal venoms contain mast cell depleting (43) enzymes (44) (proteases. phospholipase, esterases. hyaluronidase).

The *kinin-producing fractions* are mainly related to the enzymes of the venoms.

An *anaphylatoxin-producing fraction* was found in Cobra venom (45); it is known that anaphylatoxin degranulates mast cells (46, 47).

High molecular mast cell depletors have been extracted from the jelly fish (48, 49, 50, 51), from the eelworm of swine (52) and from the skin secretions of **AMPHIBIA** (53,34, 54, 55, 56).

Spermine which is known to liberate histamine (57) is found in spider venom (58). *Holothurin*, a saponin-like substance, was isolated from *Holothuria vagabunda* (59). It has been shown that surface active agents are potent mast cell depletors (60).

In Fig. 1, a simplified summary is given on the mechanism which provokes anaphylactoid shock, with special reference to the action of animal venoms.

Morphologically, mast cell degranulation by animal venoms can easily be observed. Biopsies taken at various intervals after injections of animal venoms (*Agkistrodon piscivorus*, skin secretion of *Bombina variegata*) demonstrate the different stages of mast cell degranulation. 3 hours after injection, practically all mast cells are found degranulated. The severe vascular changes at sites of injections seem to result from liberated mast cell substances as well as from direct toxic effects of the venoms (55, 61).

Mast cell degranulation by animal venoms can also be demonstrated in isolated cells by microscopic examination or by biochemical analysis of the suspension fluid (50).
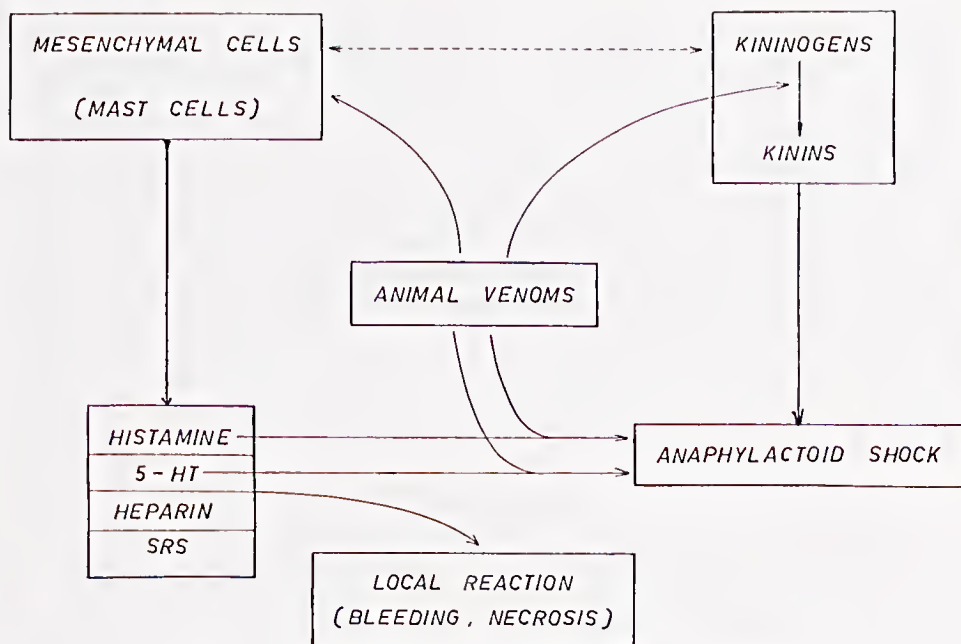
Fig. 1 — Anaphylactoid shock provoked by animal venoms.
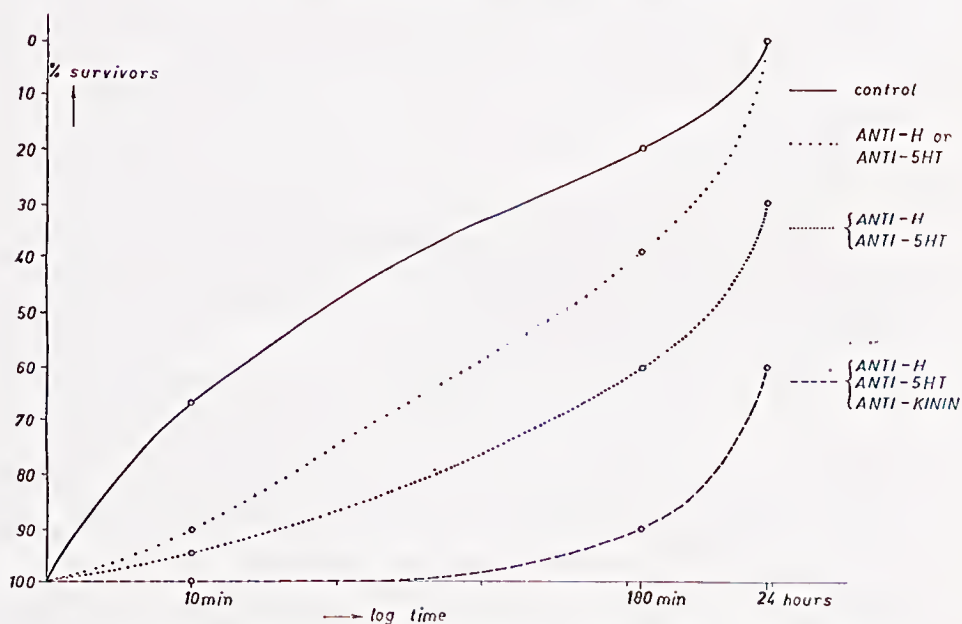
Fig. 2 — Inhibitory effect of antihistamine-, antiserotonin- and antikinin-substances on mortality following snake venom.

Anaphylactoid reactions can be prevented by administration of antamine substances: antihistamines, antiserotonins and antikinins. Antihistamines did not exert any significant influence on toxicity of snake venoms (62, 63), experi-

ments with antiserotonins and antikinins have not yet been reported. In a series of preliminary experiments, we investigated the protective action of different antamines on the toxicity of the venom of *Agkistrodon piscivorus* in mice. Pretreatment with antihistamines or antiserotonins did not show any significant influence on survival time following injection of a lethal dose of *Agkistrodon piscivorus* venom (20 mg/kg i.v.). However after administration of a combined antihistamine-antiserotonin substance (cyproheptadine), a longer survival time was found. In several animals rapid death due to anaphylactoid shock was prevented. Optimal results were obtained with a combined antihistamine-antiserotonin-antikinin substance (WA-335 Dr. Karl Thomae, Germany). A pretreatment with this substance (15 minutes before injection of the venom) did not only change survival time, but the lethal dose of the venom was tolerated by a number of mice. The high antikinin activity might exert a direct *antitoxic* effect by neutralizing toxic fractions of the venom (Fig. 2). The prevention of shock by broad spectrum anti-anaphylactoid substances might be of therapeutic value in man.

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Discussion

E. *A. Zeller:* "If serotonin, histamine, and other biogenic amines play an essential role in the action of certain venoms, then it should be possible to change the response to venom by changing the metabolism of those biogenic amines. Since amine oxidases, e.g. diamine oxidases (histaminase) and monoamine oxidases can be blocked *in vivo*, I wonder whether anybody has pretreated laboratory animals with inhibitors of the enzymes before the venoms were administered?"

E. Kaiser: "As far as I know substances blocking monoamine oxidase have not been used until now. Preliminary experiments in our laboratory have shown that pretreatment with compound 48/80 (synthetic mast cell depletor) significantly diminishes anaphylactoid reactions following snake venoms."

H. Edery: "Would you please tell us more details about this anti-kinin substance you mentioned. It is specific, it acts in other organ system reactive to kinins?"

E. Kaiser: "I am sorry to say that only very limited information is available on this substance (WA-335, Dr. Thomae, Western Germany). At the present moment I can only tell you that the substance has a high antihistamine, antiserotonin and antikinin activity. I am not informed about pharmacological tests performed by Dr. Thomae."

E. R. Trethewie: "Do you think adenosine and related enzymes released are significant in that cardiac effects? One can select a venom that is simpler in effect, e.g., *Pseudechis porphyriacus* (Australian Black Snake), antihistamine with heparine does reduce its mortality. Polyvinylpyrrolidone will prolong life with the hemotoxic Tiger Snake venom."

E. Kaiser: "Our experiments were restricted to the venom of *A. piscivorus* until now. We have no personal experience on the cardiotoxic effects of the venom."

52. MECHANISM OF HISTAMINE RELEASE BY ANIMAL VENOMS

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The ability to release histamine from tissues is a pharmacodynamic property of many animal venoms. Although not generally considered as a major cause of the lethal actions of such venoms, the hypotension and increased peripheral vascular permeability which are produced by histamine, can enhance the toxic actions of other venom components, as well as of endogenous factors released by the venom in the bitten subject. In this presentation I shall limit myself to a discussion of the biochemical aspects of the release of histamine induced by two venoms: that of the Brazilian rattlesnake *Crotalus durissus terrificus* and that of the honey bee *Apis mellifera*. The results to be presented are mostly those of our own studies, performed in collaboration with Dr. Mercedes P. de Oliveira, A. Castania, Vera Porticri and Suzana Ribeiro da Costa.

It has been known since 1956 that crotamine, a basic protein found in the venom of CROTALIDAE of southern Brazil (1), is capable of releasing histamine from rat tissues. This result was first obtained by Moura Gonçalves and Rocha e Silva (2) using the perfused hind leg preparation of the rat; the authors concluded that crotamine is the cause of the well-known histamine releasing ability of this crotalic venom. There are however, certain varieties of Brazilian rattlesnakes, more abundant in the northern and central regions of the country, which do not contain crotamine in their venoms. In a study of the pharmacodynamic properties of such venoms, we noted that they were highly active histamine releasing agents, having a potency equal or higher than that of crotamine itself. Fig. 1 shows the release of histamine from isolated rat mast cells by crotamine and crotamine-free whole rattlesnake venom. In this, as well as in most subsequent studies, histamine release was assayed on the washed mast cells isolated from the peritoneal cavity of the rat. Such cells are good representatives of the tissue-bound forms of these cells which, as it is well-known, are histamine storage sites in many species. It is by the stimulation of their granular secretion or by unspecific cytolytic damage, that histamine release is brought about *in vivo*.

It is known that the so-called hemolytic snake venoms are often powerful histamine releasing agents *in vivo* or in perfused tissues. Trethewie in Australia, has actually suggested (3) that these two activities e.g. hemolysis and amine releasing action are due to the same chemical entity. We have tried to verify whether in crotamine-free rattlesnake venom, histamine-releasing activity was associated with hemolytic or rather, to use a more precise term, phospholipase A

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activity. To this end, we made a chromatographic analysis of crotamine-free rattlesnake venom on a Amberlite Irc-50 ion-exchange column according to the technique described by Habermann (4). Fig. 2 shows the results. It can be
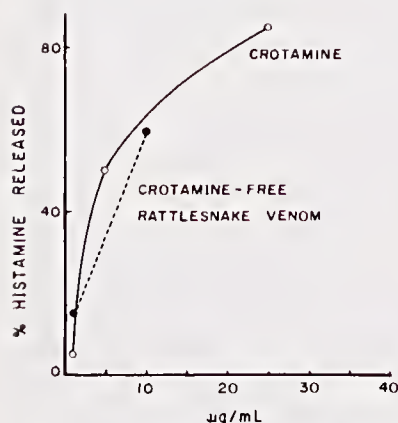
Fig. 1 — Histamine release from isolated rat mast cells by crotamine and crotamine-free whole rattlesnake venom.
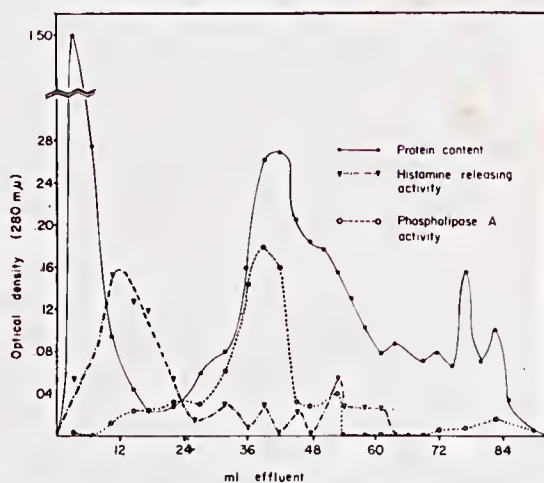
Fig. 2 — Chromatographic analysis of crotamine-free crotalic venom on Amberlite XE-64, CG-50 ionic resin.

seen that hemolytic activity, measured as phospholipase A by the egg-yolk coagulation test, could be clearly differentiated from histamine releasing activity as measured by the isolated mast cell technique. This activity was eluted shortly after, but definitely not together with crotactin (tall peak), the major protein of this rattlesnake venom, which also seems to be the major factor responsible for the lethality of such venom (4). It thus becomes clear that the histamine releasing factor of crotalic venom, which we have tentatively called enzyme fraction I, is neither crotactin nor phospholipase A. By using the combined fractions showing highest histamine releasing activity, we proceeded with an analysis of its properties. These are shown in Table I, which also compares them with

TABLE I — HISTAMINE RELEASING ACTIVITY OF CROTAMINE AND OF ENZYME FACTOR I FROM RATTLESNAKE VENOM

Treatment	Histamine releasing activity *	
	Enzyme factor I	Crotamine
Heat (5', 100ºC)	Lost	Retained
Dialysis	Retained	Lost
Digestion by trypsin	Lost	?
Electrophoretic mobility (pH 7.7)	Slow, anionic	Fast, cationic

* Assayed on rat isolated peritoneal fluid mast cells.

those of crotamine. It can be seen that we are most probably dealing with an enzyme as the thermal instability, protein nature and high molecular weight data indicate. We suspect that enzyme factor I is a proteolytic enzyme having chymotrypsin-like properties and base this assumption on three facts: a) chymotryptie activity has been demonstrated in crotalic venom by Deutsch & Diniz (5) in 1955; b) chymotrypsin is a histamine-releasing agent (6, 7) in contrast to trypsin which will not act on the isolated mast cell; c) our product was able to destroy bradykinin, a property of chymotrypsin, and which is an extremely sensitive, although not specific test, for this enzyme.

The absence of a direct histamine releasing effect of crotalic phospholipase A was a somewhat unexpected finding, even more so because of reports in the literature (Uvnäs and collaborators (8)) showing that snake or bee venom phospholipase A was indeed a potent histamine releasing agent on isolated mast cells of the rat. It seemed possible to us that the preparations used by Uvnäs could have been contaminated with other venom factors capable of releasing histamine. In order to investigate this problem, we decided to study the histamine releasing properties of a highly purified and very active preparation of phospholipase A from bee venom, obtained through the courtesy of Prof. Habermann (9) of Germany. It turned out, that this enzyme was only slightly active on the isolated mast cell, even when assayed in relatively high doses as shown in Table II. Not

TABLE II — EFFECT OF PHOSPHOLIPASE A TREATED EGG YOLK ON THE RELEASE OF HISTAMINE FROM ISOLATED RAT MAST CELLS

Releasing agent *	Percent histamine released
Phospholipase A, 20 μg/ml	7.3
Egg yolk (4%) pretreated with phospholipase A (37°, 40')	91.0
Egg yolk (4%)	0.0

* Allowed to act for 20 min, 37°C on the mast cell suspension.

unexpectedly, phospholipase A was also inactive as a hemolytic agent, an obvious conclusion from the known fact that the red cell's membrane phospholipids are shielded from attack by this enzyme (10). They are however highly sensitive to the action of the product of this enzyme's action on a susceptible substrate like egg yolk's fresh lecithin. The data on Table II indicate that rat mast cells readily loose their histamine when treated with the products of the action of phospholipase A on egg yolk, which we know to contain lysolecithin. Thus, *in vitro* release of histamine by phospholipase A can only take place in an indirect way.

The question which now arose in our minds was: would the enzyme behave in the same manner in an *in vivo* test for histamine-releasing activity? Would therefore the numerous results about the histamine releasing activity of hemolytie venoms *in vivo* or in perfused tissues demonstrated by other authors, have to be ascribed entirely to the presence of non-phospholipase A components? In order to answer these questions, we performed a very simple test for the detection of histamine releasing activity *in vivo*. This is the well-known Trypan blue skin

capillary permeability test, which consists of the following: a rat or a guinea-pig is intravenously injected with a dye like Trypan or Evans blue, which normally does not leave the circulation to enter tissue fluid spaces because of its inability to cross capillary wall permeability barriers. If such barriers are however altered by an experimental procedure such as the local release of histamine, a substance highly active on capillary permeability, diffusion of the dye across the vascular wall can take place and a blue spot will appear at the site of histamine release. Fig. 3 shows the results of intradermal injection of bee venom phospholipase A
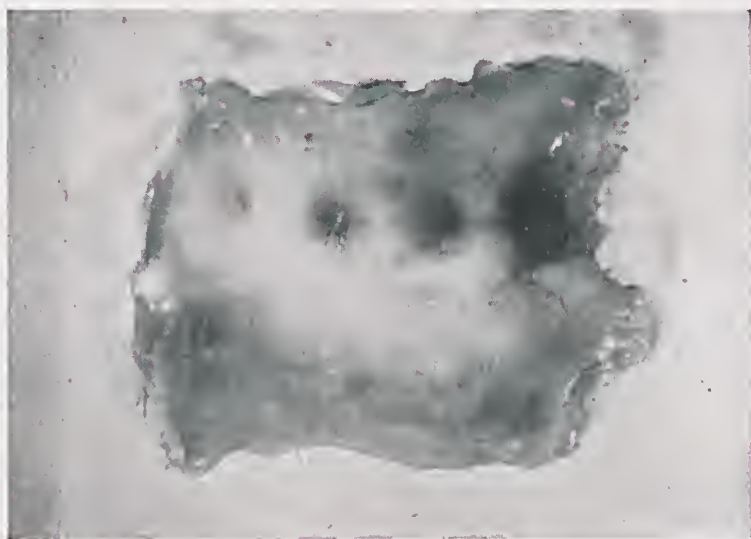
Fig. 3 — Effect of bee venom phospholipase A on capillary permeability in rat skin. Dark areas correspond to the injection of respectively 0.01, 0.1, 1.0 and 5.0 μg of enzyme. Contra-lateral injections of saline failed to produce comparable responses.

into rat skin in doses ranging from 10 μg to 5 μg. It can be seen that the enzyme was highly effective in lowering capillary diffusion barriers and that this effect bore relation to the dose used. This effect was not a direct one; it is probably mediated by the release of skin histamine and serotonin since it could be shown (13) that the permeability effects of phospholipase A could be completely suppressed in animals previously treated with a mixture of an inhibitor of the vascular effects of histamine (diphenhydramine) and an inhibitor of the vascular effects of serotonin (BOL-148, bromlysergic acid diethylamide). This latter had to be used because as it is well-known, whenever mast cell damage occurs in the rat, not only histamine but serotonin as well, is released. Serotonin is even more potent on rat capillary permeability than histamine. The obvious conclusion from these results was then, that phospholipase A was able to release mast cell amines *in vivo*, even though it proved incapable of doing the same on the isolated mast cell *in vitro*.

Table II had shown that phospholipase A can induce copious histamine release from isolated mast cells *in vitro* but only in an indirect way, namely, through the formation of lysolecithin. As it is well-known, this substance, a highly cytolytic agent, is the result of the splitting of an acid radical from lecithin by

phospholipase A. Could this reasoning explain the potent effects of the enzyme on rat skin *in vivo*? We believe this to be so. Firstly, because tissue fluids, specially lymph, are known to contain phospholipids which could function as substrates for phospholipase A, yielding cytolytic phosphatides in the *in vivo* situation; secondly, because we have actually been able (13) to show a significant, even though small, histamine releasing action of the bee venom enzyme on rat skin *in vitro*. To our understanding, these results indicate that histamine release by phospholipase A is always an indirect process. It can take place in rat skin because this tissue, even *in vitro*, possesses enough free phospholipid to serve as a source for lysolecithin; in contrast, free, washed mast cells have no other phospholipid besides that contained in their cell membranes or cytoplasma. This phospholipid material must be shielded from the direct attack by even as potent a phospholipase A as is the bee venom enzyme.

Before finishing this presentation, I would like to show a few photomicrographs illustrating the effects of histamine-releasing compounds on rat mast cell morphology. Fig. 4a shows the typical, toluidine blue stained mast cell of rat mesentery spreads, with its metachromatically stained, heavy population of granules which, besides heparin, contain all of the mast cell's histamine. Fig. 4b shows the unchanged appearance of the mast cell, following incubation with 25 μg/ml of phospholipase A. When phospholipase A-treated egg yolk was added to the mesentery fragment however, distinct signs of cell degranulation could be observed (Fig. 4c). This phenomenon, of mast cell degranulation, occurs whenever histamine release takes place. It probably represents the primary response of mast cells to
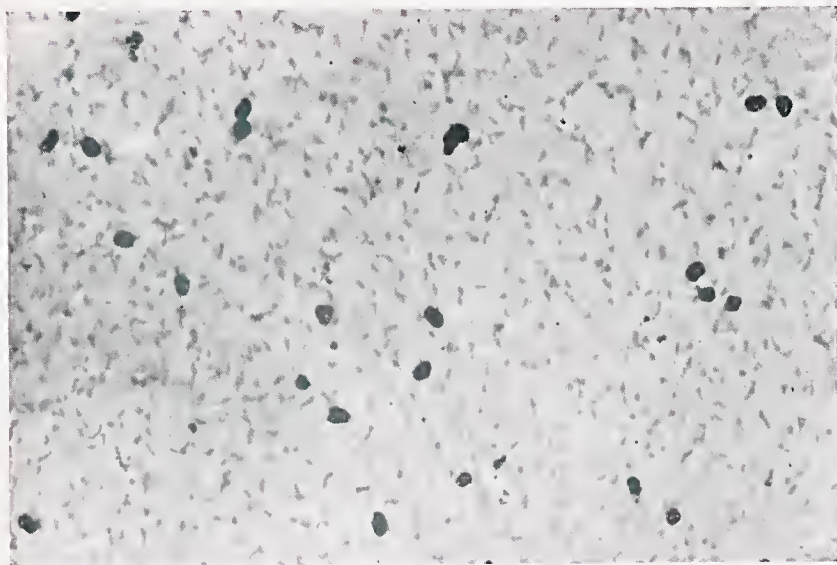
Fig. 4a

Fig. 4 — *In vitro* morphological alterations of rat mesentery mast cells by bee venom phospholipase A, mellitin and compound 48/80. a) Untreated controls; b) incubated for 25 min at 37° C with 25 μg/ml of phospholipase A; c) incubated for 25 min with the product of the action of phospholipase A (25 μg/ml) on 4% egg yolk; d) incubated for 10 min with cp. 48/80 (5 μg/ml); e) incubated for 25 min with 25 μg/ml of mellitin.
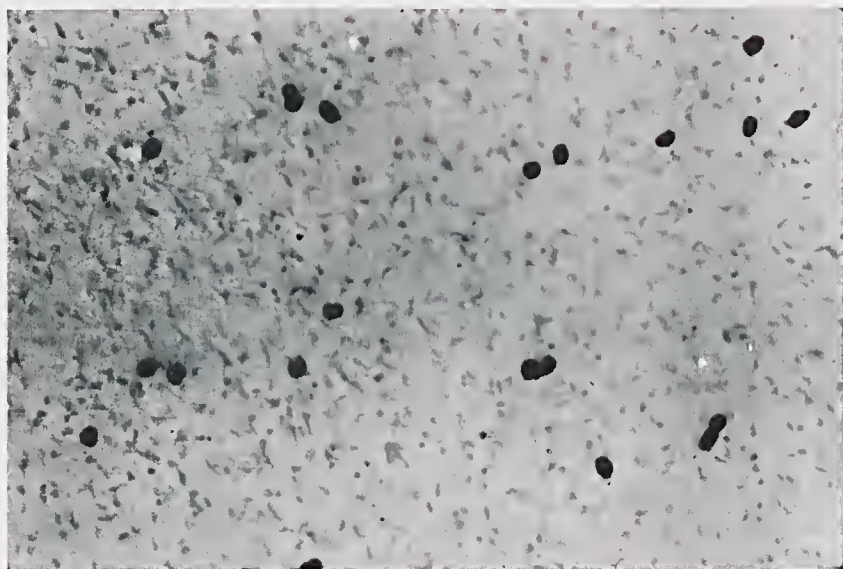
Fig. 4b
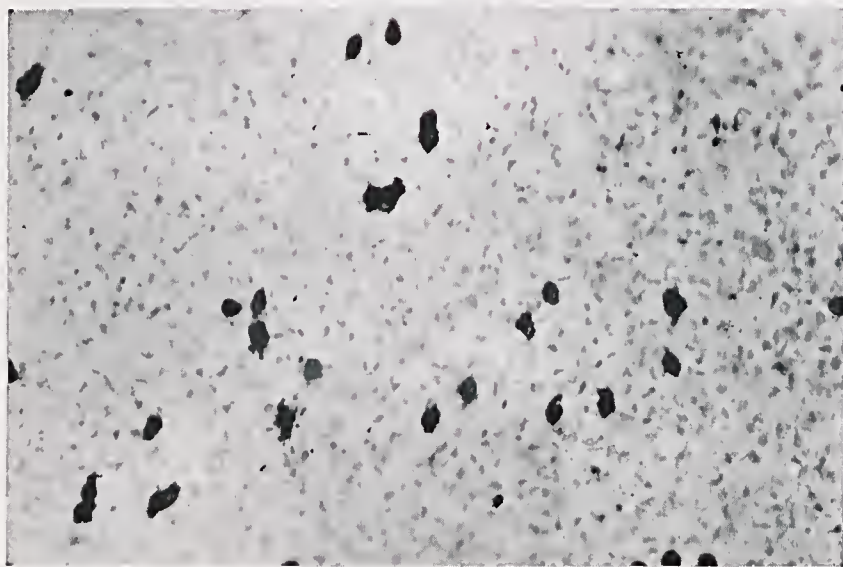
Fig. 4c
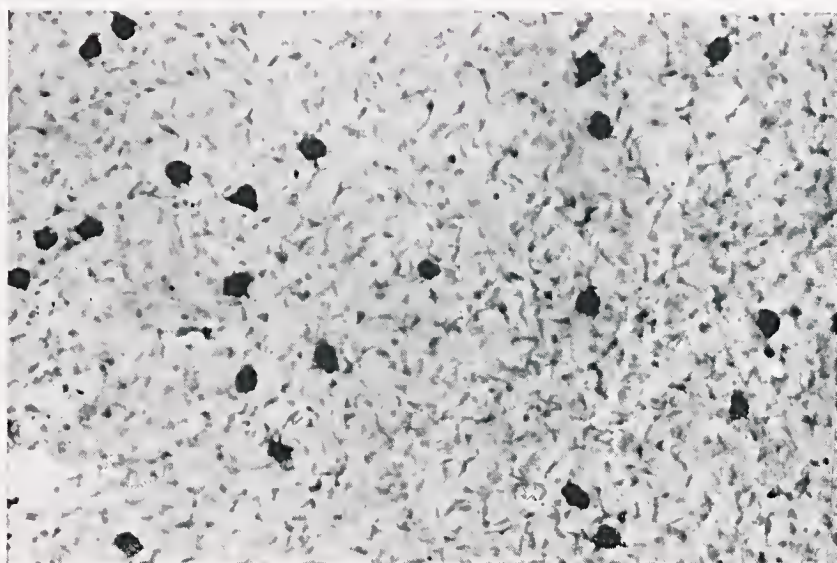
Fig. 4d
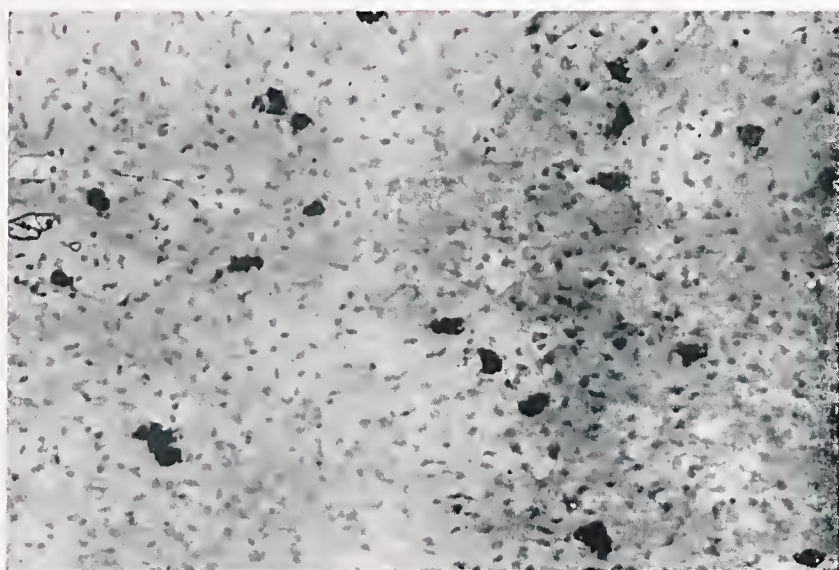
Fig. 4e

histamine releasing stimuli and it is likely, that it is only in the shed granules that histamine breaks loose from its intracellular ties, becoming free to exert pharmacological actions in the body. Fig. 4d shows, for the sake of comparison, the effect of compound 48/80, the most potent chemical histamine releaser in the rat known, on the mast cells of another sample of mesentery. Fig. 4e shows

the effects of melittin; this is a polypeptide substance, isolated from bee venom by Habermann (14), which has an intense and apparently unspecific cytolytic effect. It induces 100 per cent release of histamine from isolated mast cells (13). As it can be seen, its effects are not so much a degranulation but rather a dissolution of the cell's boundaries. This aspect is typical of unspecific cell damage. and is, in its mechanism of action quite different from granule secretion as evoked by compound 48/80 or the antigen-antibody reaction. The action of melittin is however similar to that of lysolecithin. Table III shows that there is a means

TABLE III — MODES OF ACTION OF HISTAMINE RELEASING AGENTS

Group I: Stimulants of mast cell secretory activity (effects inhibited by metabolic inhibitors (anoxia, DNP, NaCN)

- Antigen-antibody reactions
- Epinephrine
- Compound 48/80
- Curares
- Chymotrypsin
- Enzyme factor I from crotalic venom
- Crotamine

Group II: Non-specific cytolytic agents (effects *not* inhibited by metabolic inhibitors)

- Surfactants (octylamine, Tween 20, etc.)
- Phospholipase A
- Lysolecithin (egg yolk)
- Melittin
- Crotamine

of distinguishing between the mechanism of action of histamine releasing agents. I consider those agents whose action is inhibited by metabolic inhibitors like oxygen lack, dinitrophenol or cyanide, to be true stimulants of the secretory machinery of the mast cell. In contrast, the group of histamine releasers whose action is not blocked by metabolic inhibitors, are to be considered unspecific cytolytic agents, capable of rupturing the mast cell in the same way as they would injure most any cell in the mammalian organism. It can be seen that among histamine releasing agents contained in animal venoms, phospholipase A, melittin and, to a partial extent, crotamine, are included in this group.

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Discussion

F. Kornalík: "How do you explain the fact, that ELAPIDAE venoms, especially the venom of *Naja naja* which are known to have big amounts of phospholipase A are not able to liberate any histamine from the tissue? Have you tested the phospholipase A from Prof. Habermann in another way than the skin test for the presence of spreading factor?"

A. M. Rothschild: "I have no personal experience with such venoms. However the statement that the venom of *Naja naja* does not release histamine from tissues contradicts observations reported by a considerable number of authors who have found this venom to be quite powerful as a histamine releasing agent. We have not done a characterization of this enzyme because Prof. Habermann (9) has presented convincing proof that it is essentially free of enzymatic contaminants including hyaluronidase. Furthermore, our skin tests definitely implicate histamine and serotonin as mediators; hyaluronidase is known not to release these amines."

J. L. Prado: "The bradykinin destroying effect of enzyme I fraction of snake venom seemed too slow to me; would it really be a chymotryptic enzyme?"

A. M. Rothschild: "There is, I believe, fair evidence that the histamine releasing activity of Fraction I is of an enzymic nature. Although our results do not conclusively characterize this activity as being chymotrypsin-like, they point in this direction. Slugishness of action does not necessarily rule out this possibility since even whole crotalic venom is slow in attacking a typical chymotryptic substrate like ATEE."

E. A. Zeller: "Have you tested the substrate pattern of your enzyme factor which you suggested appears to be a chymotrypsin-like proteinase?"

A. M. Rothschild: "No."

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53. USE OF VENOMS IN STUDIES ON NERVE EXCITATION

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INTRODUCTION

Various techniques have been utilized over the past several decades in attempting to understand bioelectrogenesis, the primary event in neural activity. Utilizing electrophysiological techniques it was possible to analyze many aspects of electrical activity of conducting tissues (1, 2). However, for elucidating the chemical and molecular basis of nerve activity it was necessary that techniques of modern biochemistry and pharmacology be used. Over the past 25 years the studies of Nachmansohn and coworkers have provided us with the essential steps involved in the generation of bioelectricity although many details require further investigation (3-5).

The impetus which led to our studying of the effects which structure and organization of biological membranes and their environment have on the functioning of the ACh system, and on the action of compounds applied externally to biological tissue, will be better understood if we briefly review the role attributed to acetylcholine (ACh) in the excitable membrane during nerve conduction (Fig. 1). ACh in resting condition is present in a bound form. Any stimulus reaching the membrane leads to release of the ester which combines with a receptor. In the reaction of ACh with the receptor a change in conformation of the receptor is thought to occur which leads to a shift of charge initiating the permeability changes of the membrane associated with the passage of a nerve impulse, i.e. sodium ions enter the axon and potassium ions leave. The rapid inactivation of the ester by acetylcholinesterase (AChE) permits the receptor to return to its resting condition. A prerequisite for this theory was the demonstration of the presence in all conducting tissue of choline acetylase (ChAc) the enzyme which synthesizes ACh and AChE the enzyme which hydrolyzes ACh. Of crucial importance to the development of the theory has been, however, the evidence that block of either AChE or ChAc by potent or competitive inhibitors leads inevitably to block of electrical activity.

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Recipient of a Public Health Service Research Career Development Award, 5-K3-NB-21, 862.
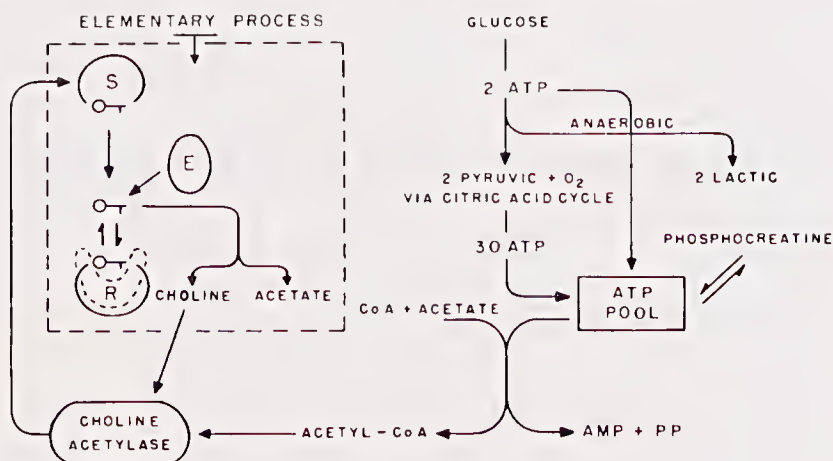
Fig. 1

Some investigators have, however, not accepted the theory that ACh has an essential role in axonal conduction, and have maintained that ACh is essential for transmission of impulses only at certain junctional regions. One of their major objections to Nachmansohn's theory was the failure of ACh, and other quaternary nitrogen derivatives to affect conduction in nerve axons in contrast to their powerful action on junctions. The excitable and conducting axonal membranes, in contrast to those at synaptic junctions, are surrounded by structures rich in lipid material, the Schwann cells, which form a permeability barrier preventing lipid insoluble compounds from reaching the membrane. Experimental evidence for the existence of barriers was obtained by the demonstration that both ACh and the quaternary cholinesterase inhibitor neostigmine, failed to penetrate into the axoplasm of the squid giant axon after their application to the fiber in the external solution, whereas lipid soluble tertiary nitrogen derivatives, such as physostigmine, that blocks electrical activity, were found in the axoplasm (6, 7). In preparations such as the rabbit vagus and axons from the lobster walking leg in which the permeability barriers are apparently incomplete, direct effects on conduction have been obtained with ACh. and related compounds, such as d-tubocurarine (curare) known to compete with ACh for receptor sites at junctions (8-11).

If ACh and curare are inactive on the squid giant axon and other axonal preparations because of permeability barriers it appeared possible that by chemical treatment one could decrease these barriers and then demonstrate actions of lipid insoluble compounds which interact with the ACh system. After exposure of the frog sciatic nerve to a detergent, axonal conduction was reversibly blocked by ACh, curare, neostigmine and other quaternary nitrogen derivatives (12). In our studies we elected to use the squid giant axon (Fig. 2) because it is a large single fiber and is non-myelinated, i.e. is surrounded by a relatively thin Schwann cell. In such a preparation chemical treatment might be expected to be more effective and better controllable than in a multifibred preparation. This preparation also has the unique advantage that its axoplasm can be readily extruded in sufficient amounts to allow penetration studies to be carried out. In addition it was useful to study a preparation for which a considerable amount of data are

already available, and with which our results could be correlated. This review shall present and summarize some of the findings we have made over the past 5 years (14-25).
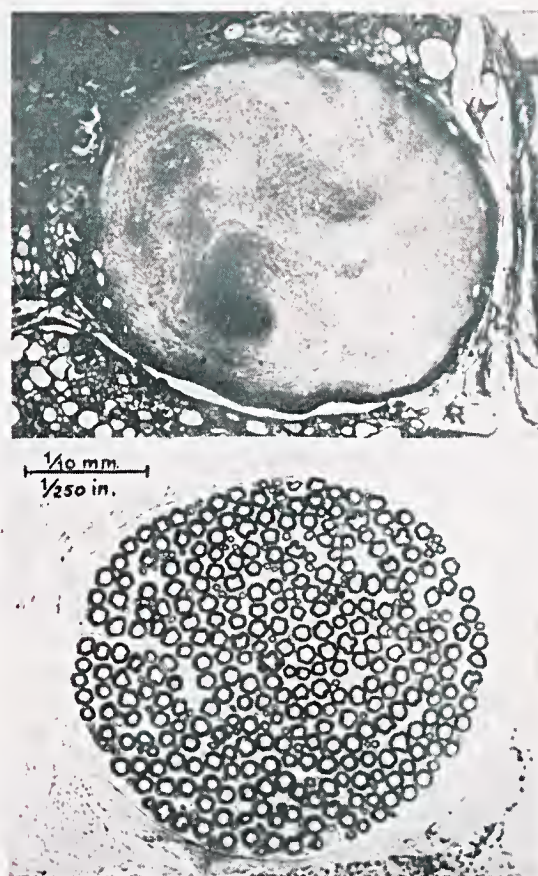
Fig. 2

Effects of tertiary and quaternary nitrogen derivates on conduction

While a few pharmacological agents have been tested on conduction of the squid giant axon, no systematic comparison had been made between tertiary and quaternary nitrogen containing compounds. If a permeability barrier prevents lipid insoluble quaternary nitrogen compounds from reaching the conducting membrane, then we should find these compounds to be inactive on the squid axon even if they interact with the ACh system at junctions where the permeability barriers are much weaker. We should also find that lipid soluble compounds which affect junctional transmission should also affect axonal conduction. This is what we did find on control squid axons in Table I where we have also compared the potency of various compounds on the squid axon with that observed at the synaptic junctions of the isolated single electroplax where barriers are either minimal or absent, and where approximate affinity to receptors present

may be determined (28, 29). The tertiary nitrogen derivatives are effective in very similar concentrations on these two preparations. One could not, however, expect exact agreement, even if the permeability barriers were completely absent, because of species differences, somewhat different experimental conditions and most likely other factors involved. In contrast all quaternary nitrogen containing compounds except noracetylcholine and pyridine aldoxime dodecyliodide (PAD) are ineffective on the squid axon in contrast to their high potency at the synapse. Noracetylcholine and PAD are lipid soluble derivatives of acetylcholine and pyridine aldoxime methiodide (PAM) having a dodecyl group which replaces a methyl group on the nitrogen. We shall discuss the results obtained on the venom treated squid axon in section 3. ACh and curare were equally inert on control squid axons which were very carefully dissected free of all adhering small nerve fibers as well as axons in which not all of the small nerve fibers were removed (23). Physostigmine was also found to be almost equally potent in both preparations. These results indicate that the adhering small nerve fibers and associated connective tissue do not constitute a strong permeability barrier. The major barrier is apparently the Schwann cell surrounding the giant axon. For convenience we have therefore usually used preparations in which no special effort was made to remove all adjacent fibers, although as will be discussed in sections 3, 4 and 8 venom effects and penetration of ACh were compared with both types of preparations.

These results show that lipid soluble compounds expected to interact with the ACh system affect conduction along the axon as well as at the junction, whereas lipid insoluble compounds block junctional transmission but not axonal conduction.

Venom pretreatment of squid giant axon

In attempting to reduce the permeability barriers surrounding the squid giant axon, various enzymes, detergents and other compounds in the highest concentrations which had no effect on conduction were applied to the axon for 30 minutes. After removal of the pretreatment agent and rinsing in normal sea water for 15 to 30 minutes, 1.4×10^{-3} M curare was applied for 30 minutes. Curare was used as a test compound for indicating disruption of permeability barriers since it is highly potent at synapses and is not readily metabolized by enzymes of biological tissue such as for example ACh is by AChE. Curare had no effect on conduction following exposure of the axon to digitonin, hyaluronidase, trypsin, chymotrypsin, hydrolase mixture, lipase, lysozyme, papain, alkaline phosphatase, sodium desoxycholate, Span 20, Tween 20, neuraminidase, saponin, arsenite, dimethyl formamide, dimethyl sulfoxide or histamine (14, 24). Following pretreatment with *Naja naja* (hooded cobra) snake venom, however, curare was found to reversibly block conduction (14). An axon rendered sensitive to the action of curare remained so over a long period of time. For example curare was equally effective whether pretreatment was followed by placing in sea water for 10 min. or 95 min. (Fig. 3). This figure shows effects obtained with a combination of hooded cobra venom plus a detergent, however, similar results were obtained with venom alone. Since snake venoms appeared to render the axons sensitive to curare a systematic study of the effects of curare following venom pretreatment of the axon was undertaken. Of a series of 21 venoms tested *Agkistrodon p. piscivorus* (Cottonmouth moccasin) venom was most effective in rendering the axon sensitive to curare (Table II). Examples of the effects of curare

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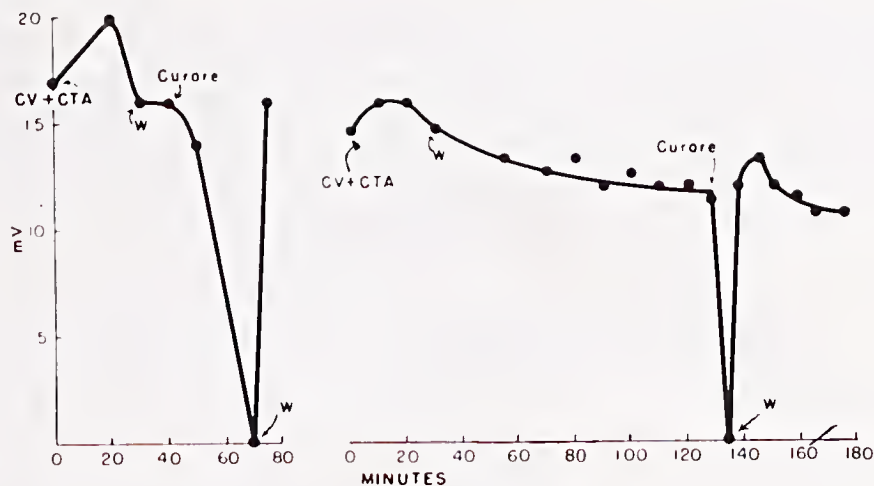


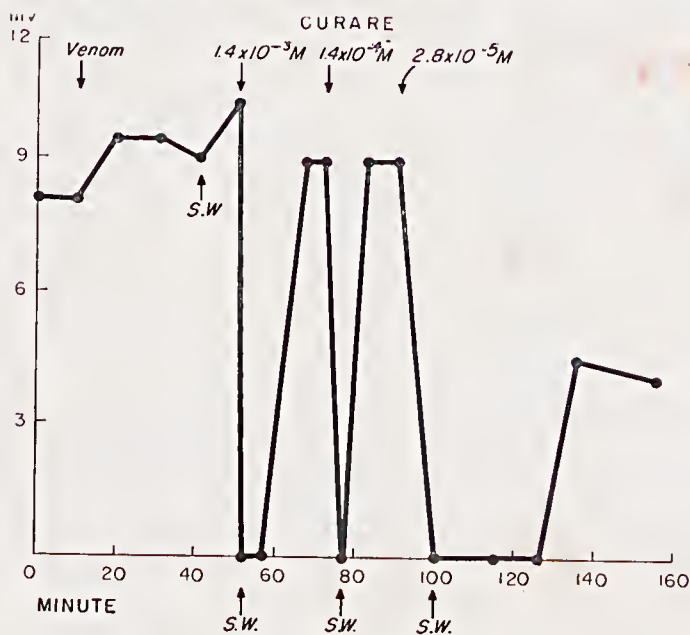
Fig. 3

TABLE I — MINIMAL CONCENTRATIONS OF SEVERAL COMPOUNDS REQUIRED TO BLOCK CONDUCTION OF CONTROL AND VENOM TREATED SQUID GIANT AXONS AND SYNAPTIC TRANSMISSION IN THE ISOLATED SINGLE ELECTROPLAX

Compounds were applied for 30 minutes. Venom treated axons were pretreated for 30 minutes with 15 μg/ml *Agkistrodon p. piscivorus* venom. > indicates that at concentration shown no effect on conduction was observed. Noracetylcholine = β-acetoxyethyl-dimethyldodecyl ammonium iodide; PAD = Pyridine aldoxime dodecyliodide; PAM = 2-Pyridine aldoxime methiodide.

COMPOUND	[M] TO BLOCK CONDUCTION		
	Squid Axon *		Electroplax Synapse **
	Control	Venom Treated	
Tertiary Nitrogen Derivatives			
Atropine	2×10^{-3}	3×10^{-4}	3×10^{-4}
Methantheline	2×10^{-3}	—	5×10^{-4}
Physostigmine	7×10^{-3}	1×10^{-3}	7×10^{-4}
Procaine	3×10^{-3}	—	1×10^{-3}
Dibucaine	3×10^{-5}	—	3×10^{-5}
Diphenhydramine	4×10^{-4}	—	2×10^{-4}
Chlorpromazine	1×10^{-4}	—	1×10^{-4}
Quaternary Nitrogen Derivatives			
Acetylcholine	$> 10^{-1}$	2×10^{-4}	3×10^{-6}
Curare	$> 10^{-2}$	3×10^{-5}	3×10^{-6}
Decamethonium	$> 10^{-1}$	2×10^{-3}	3×10^{-6}
Benzoylcholine	$> 10^{-1}$	2×10^{-2}	1×10^{-3}
Chlorisondamine	$> 10^{-1}$	1×10^{-2}	2×10^{-4}
PAM	$> 10^{-1}$	1×10^{-2}	—
PAD	5×10^{-4}	—	5×10^{-5}
Noracetylcholine	2×10^{-4}	—	2×10^{-5}
Choline	$> 10^{-1}$	$> 10^{-1}$	$> 10^{-1}$

* Data from Rosenberg *et al.* (14-16).
** Data from Rosenberg *et al.* (26, 27).

following cottonmouth moccasin venom are shown in Figs. 4 and 5. In the concentrations used for pretreatment the venoms had no effect on the action potential. Hooded cobra venom depolarizes the lobster axon (30), but it is not apparent whether depolarization is the cause of the conduction block or occurs subsequent to the block. On the squid axon the resting and action potentials

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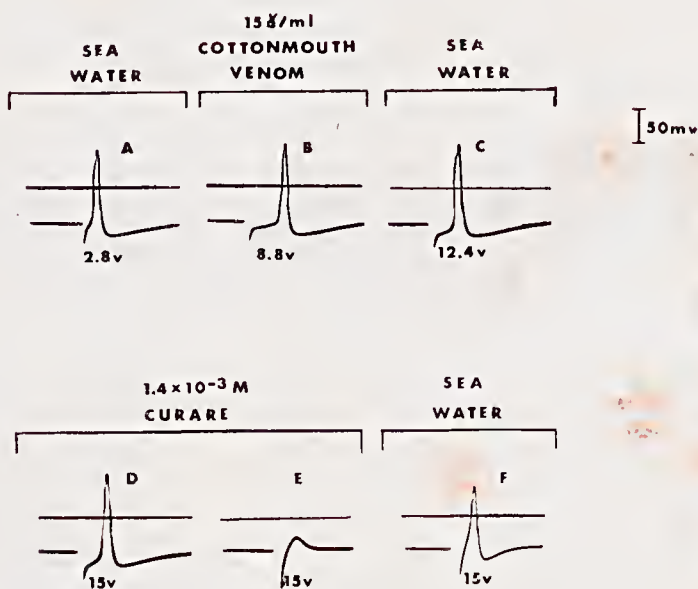
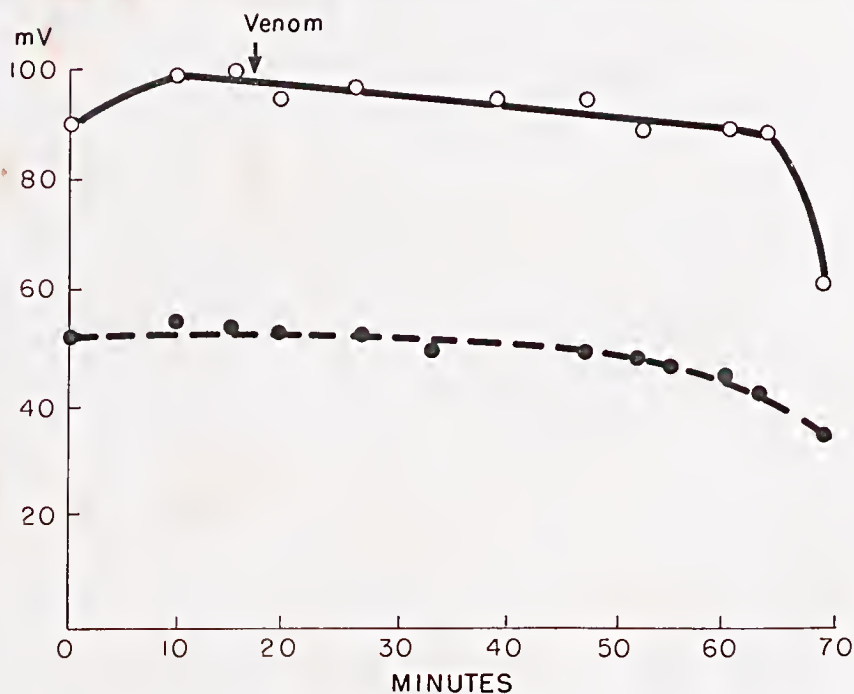


Fig. 5

were found to decline simultaneously (Fig. 6), whereas after cottonmouth moccasin venom, depolarization occurred only after the action potential was markedly reduced (Fig. 7). There was no difficulty of determining whether block of conduction was due to curare or venom since the effects of the venoms on the action potential were always irreversible whereas conduction block by curare was usually readily reversible. Curare had little or no effect on the resting potential after venom pretreatment (Fig. 5) which agrees with its effects at junctions where

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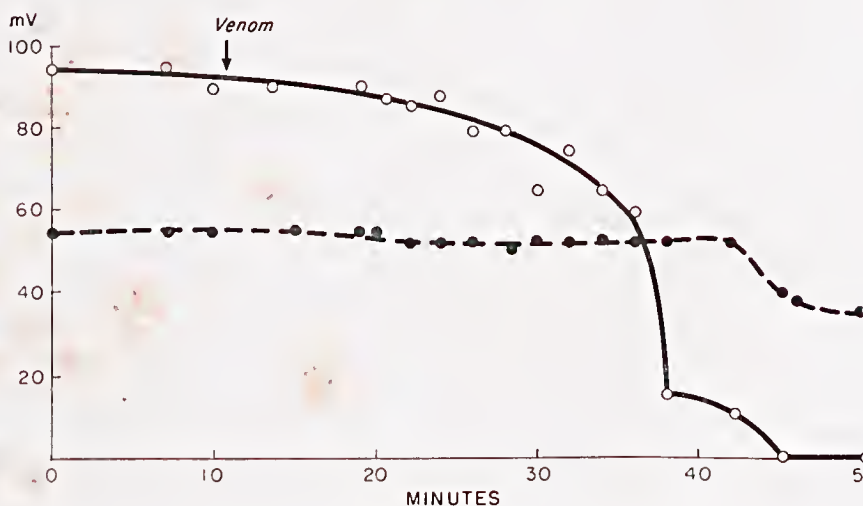


Fig. 7

it also blocks conduction but does not depolarize. It apparently interacts with the receptor to block the effects of ACh, but does not cause the change in conformation of the receptor responsible for altered conductance. It may be called a receptor inhibitor in contrast to compounds such as ACh which depolarize and may be classified as receptor activators.

The ability of the snake venoms to render curare active almost exactly correlates with their direct potencies on electrical activity of the squid giant axon (Table II) indicating that the same venom component may be responsible for

TABLE II — CONCENTRATIONS OF VENOM PRETREATMENT WHICH RENDERED THE SQUID GIANT AXON SENSITIVE TO CURARE, AND CONCENTRATIONS REQUIRED TO BLOCK DIRECTLY THE CONDUCTED ACTION POTENTIAL (AP). VENOMS WERE APPLIED FOR ABOUT 30 MINUTES

> indicates that at concentration shown no effect on AP was observed. +++, ++, +, and — indicate respectively a 80-100%, 50-80%, 20-50% and 0-20% decrease in the AP produced in 30 minutes or less by 1.4×10^{-3} M curare after venom pretreatment. The effect of curare was readily reversible. Results taken from Ref's 14-16, 24.

VENOM	μg/ml		Curare effect
	Direct block of AP	Pretreat.	
Agkistrodon p. piscivorus	50	15	+++
Notechis scutatus	100	30	+++
Acanthophis antarcticus	100	30	++
Naja naja	100	30	++
Heloderma horridum	100	30	++
Heloderma suspectum	100	30	++
Enhydrina schistosa	100	20	—
Dendroaspis polylepis	100	30	+
Bungarus coeruleus	250	50	++
Ophiophagus hannah	400	100	++
Crotalus atrox	500	150	+
Agkistrodon c. mokeson	1000	200	+
Bothrops atrox	1000	200	+
Bitis arietans	2000	500	++
Vipera russellii	> 1000	500	+
Centruroides sculpturatus	> 100	100	—
Vespula arenaria	> 100	100	—
Latrodectus geometricus	> 100	100	—
Latrodectus varidus	> 250	250	—
Crotalus h. horridus	> 500	500	—
Crotalus adamanteus	> 2000	2000	—

both effects. Studies by others of venom effects at the neuromuscular junction (31, 32) have also shown cottonmouth venom to be more potent than *Crotalus adamanteus* (Eastern diamondback rattlesnake) venom, which in our studies was one of the least effective of the snake venoms. Likewise the relative effectiveness of hooded cobra and rattlesnake venoms in our studies and in causing demyelinating changes in the central nervous system (33, 34) are similar. In contrast rattlesnake venom is more toxic than cottonmouth moccasin venom (35,

36). It is especially interesting that the hemolytic and hemagglutinin activities of several snake venoms as found by Minton (35) is in excellent agreement with their abilities to render curare active. For example he reports that Eastern diamondback rattlesnake venom has little or no hemolytic activity whereas *Crotalus atrox* (Western diamondback rattlesnake) venom is more potent which also agrees with our findings. He also found *Agkistrodon c. mokeson* (copperhead moccasin) to be less potent than cottonmouth moccasin. The marked similarity between his and our results may indicate that the same venom component is responsible for both effects measured.

Since cottonmouth moccasin venom appeared most effective in rendering the axon sensitive to curare it was selected for additional studies. The venom treated axon not only became sensitive to curare but as shown in Table I, ACh, decamethonium and several other quaternary nitrogen derivatives blocked conduction.

Examples of the ACh effects on cottonmouth venom treated axons are shown in Figs. 8 and 9. As can be seen in Fig. 9 the effects of ACh were not always reversible. In a large series about 1 out of 4 experiments with ACh were reversible. In experiments with intracellular electrodes, 4×10^{-2} M ACh following venom pretreatment blocked conduction and decreased the resting potential about 16% in 30 min. while in control axons ACh had no effect on the resting or action potential. However, 4×10^{-3} M ACh following venom pretreatment had a marked effect on the action potential but no apparent effect on the resting potential. This is in contrast to other preparations where the effects of ACh on the action potential are paralleled by concurrent effects on the resting potential (9-11).

The concentrations of quaternary nitrogen derivatives shown as being effective in venom treated axons (Table I) are the minimal concentrations which in at least a few experiments blocked conduction. For fairly consistent effects it was necessary to use concentrations 5 to 10 fold higher. The concentration of quaternary nitrogen derivatives listed in Table I as being effective on the venom treated axon are 10 to 1000 fold greater than those required at the synapse. However, at least part of this difference is quite obviously due to the incomplete reduction of permeability barriers surrounding the axon, so that only a relatively small fraction of the externally applied ACh, curare etc. is able to penetrate (see section 4). The inactivity of choline in venom treated axons, despite the fact that it penetrates under these conditions (see section 4) show that the effects of ACh are specific. At those synapses where ACh is highly potent, such as for example the electroplax synapse, it has also been found that choline is inert (Table I). In contrast to the quaternary compounds shown in Table I which were effective on venom treated axons carbamylcholine and neostigmine even in 5×10^{-2} M concentrations were inactive. These compounds are apparently even more lipid insoluble than ACh, curare, or choline since neostigmine does not penetrate into the squid axon under conditions where the venom markedly increased the penetration of the other compounds (section 4). In these experiments we observed only blocking effects of ACh and not any electrogenic action. With the method of application used, one cannot expect to mimic the suggested physiological action of ACh. Physiologically ACh would be liberated within a membrane of about 80 A° thickness. It would then act, in microseconds, on a protein receptor in close proximity, probably only a few A° away. Whereas this action may be very efficient and rapid, ACh when applied externally must penetrate through a relatively large amount of tissue before reaching the receptor of the active axonal membrane. Thus, the conditions are not comparable.

15
mV
Venom
S.W
ACh
4.4x10-2M
10
ACh
4.4x10-1M
5
S.W
ACh
4.4x10-3M
0
20
40
60
80
100
120
140
160
MINUTES
S.W.
S.W.

Fig. 8

15 γ/ml
SEA WATER
COTTONMOUTH VENOM
SEA WATER
A
B
C
50mv
2.9v
3.8v
3.9v
4.4x10-3 M
ACETYLCHOLINE BROMIDE
SEA WATER
D
E
F
4.3v
4.6v
5.5v

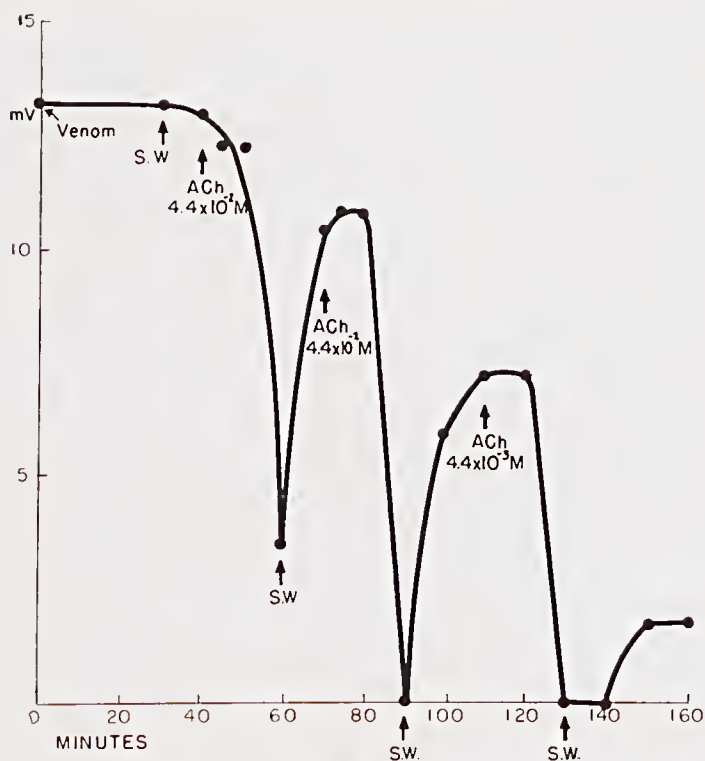
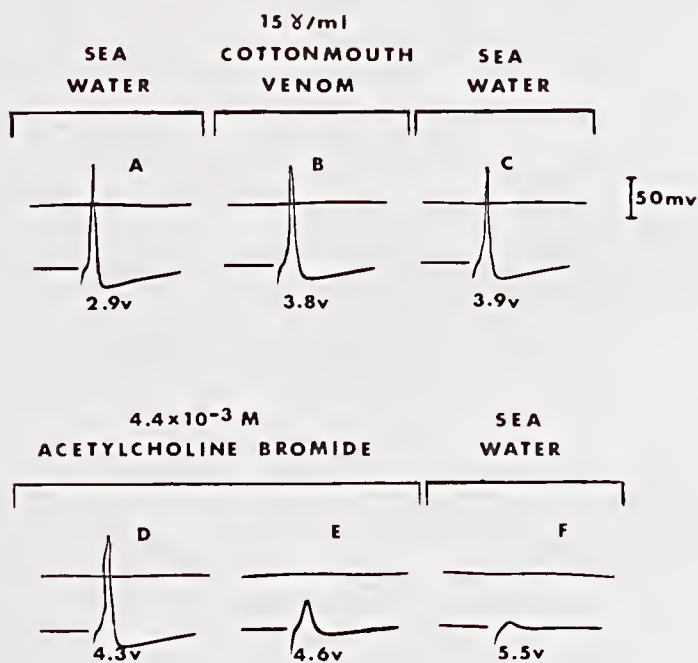



Fig. 9

It was found (Table I) that the potency of atropine and physostigmine, tertiary nitrogen derivatives, was also increased by venom pretreatment so that the concentrations effective on the axon become very close to those effective at the electroplax synapse. These findings are in agreement with the idea that venoms are disrupting permeability barriers, since even relatively lipid soluble materials probably cannot freely diffuse through the membrane surrounding the giant axon. The findings with atropine, physostigmine and the other compounds shown in Table I appears to establish that a functional ACh receptor and AChE are present in the squid axon.

Effect of venoms on permeability of squid giant axon

We interpreted the demonstration that ACh and curare affect electrical activity after exposure of squid giant axons to cottonmouth venom as being due to a reduction of the permeability barrier which prevents lipid insoluble compounds from reaching the conducting membrane. In view of the crucial question involved, viz. whether ACh and curare act on the receptor in the axonal membrane, this interpretation was submitted to a direct test; i.e., whether the venom which has been shown to allow ACh and curare to affect electrical activity also allows them to penetrate.

Axons were exposed to solutions of venom or in the case of control axons to normal sea water for 30 min., followed by a 10 min. washing and by a 60 min. exposure to the C^{14} labelled compound being studied. The axoplasm was then extruded and C^{14} assayed. A summary of results obtained are shown in Fig. 10. These data clearly established that ACh and curare do penetrate after treatment with the low concentrations of cottonmouth moccasin venom which renders these compounds active. In contrast after exposure to even high concentrations of Eastern diamondback rattlesnake venom, curare neither penetrates nor does it affect electrical activity. In control axons the penetration of ACh, choline and dimethylcurare is less than 1 per cent of that expected if no barrier were present. Even these low values however may not represent actual penetration but may be due to contamination during the process of extrusion, or the presence of trace amounts of C^{14}-tertiary nitrogen containing compounds or trace impurities in the radioactive samples of the quaternary compounds. This suggestion is supported by the finding that the penetration of ACh in venom treated axons appears to increase with increasing time of ACh incubation, whereas in axons not pretreated with venom the apparent ACh penetration after 5 min. incubation is as great as after 60 min. The amount of contamination occurring during extrusion would be expected to remain approximately constant regardless of whether the ACh incubation time was 5 or 60 min.

We found the penetration of ACh to be equally low both in axons dissected free of all adhering small nerve fibers as well as in axons containing much connective tissue and surrounding small nerve fibers. This agrees with our findings that ACh had no effect on conduction in both types of preparations (23), but is in disagreement with a report that ACh rapidly enters the axoplasm of finely dissected axons (37). In that study, moreover, the electrical activity of the axons was not checked, which makes the meaning of the experiments questionable. In contrast to the quaternary compounds the lipid soluble tertiary nitrogen derivative trimethylamine readily penetrated (Fig. 10) even without venom treatment which confirms an earlier report (6).

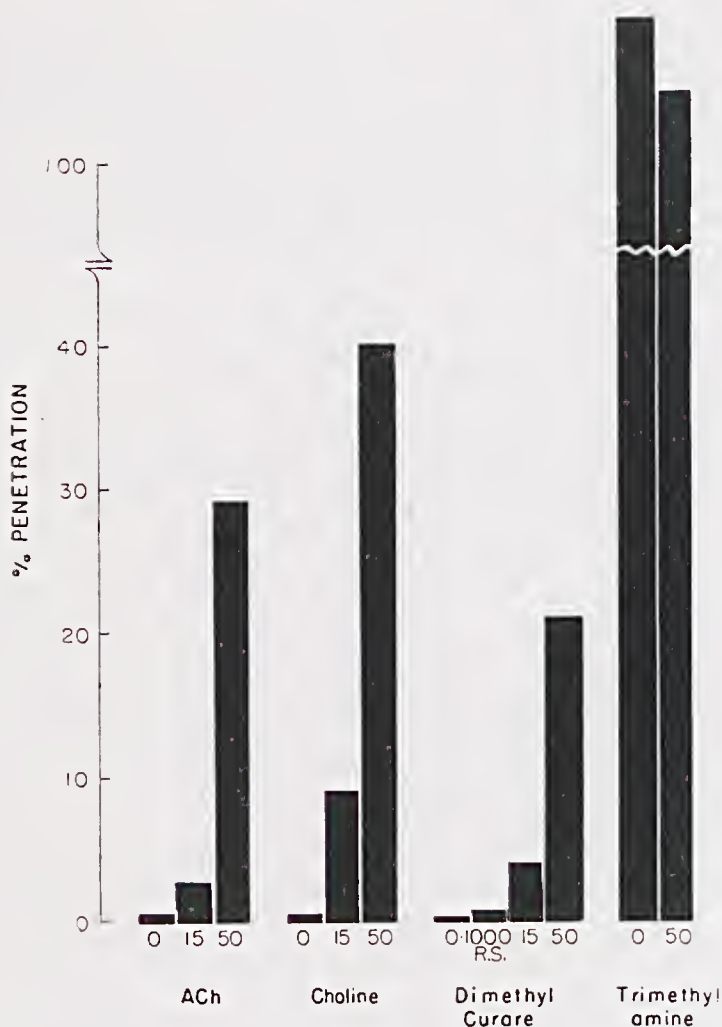



Fig. 10

In the studies described radioactivity has been measured in the axoplasm, although our interest is in knowing the concentration of the compounds reaching the active sites of the membrane which are surrounding the axoplasm. Although only about 80 A° thick (38, 39), the membrane is complex and the exact location of the ACh system is not known. It is impossible to know what the concentration of ACh or curare may be in the membrane compared to that found in the axoplasm. Even the meaning of concentration in such a membrane is uncertain. Considering the wide variety of compounds to which nerve axons during their entire length may be exposed in the body, one would expected that the receptor areas of the excitable membrane may be especially well protected against the action of external compounds. Measuring the concentration of the compounds tested in the whole axon would be of questionable significance, since there is non specific binding of charged molecules such as ACh and curare with many macromolecules in the Schwann cell, i.e., external to the active neuronal membrane (23).

Since we had found that the powerful ChE inhibitor neostigmine does not affect conduction even after venom pretreatment, we measured its per cent penetration into the axoplasm using a magnetic diver device (21). On control axons or after treatment of the axon with 25 μg/ml cottonmouth moccasin venom only 0.1 per cent penetration of neostigmine was observed, while after 100 μg/ml of venom, a concentration which blocked conduction, 1 per cent penetration was observed. The poor penetration of neostigmine therefore appears to be in good agreement with its ineffectiveness in blocking electrical activity. Neostigmine thus is apparently even more lipid insoluble than ACh or curare.

The increased permeability produced by the venoms agrees with suggestions made many years ago, even before any experimental evidence was available, that many effects of venoms are due to increased permeability of biological membranes. More recently it was found that moccasin venom increases the passage of perfusion fluid through frog atria (40) and the penetration of procaine into frog sciatic nerve (41). Cobra venom releases ACh from binding particles of the central nervous system (42) while cottonmouth moccasin venom causes the release of glutamine oxalacetic transaminase from certain rat tissue (4). Eastern diamondback rattlesnake venom which is ineffective in our studies was also unable to disrupt rabbit liver microsomes in contrast to another venom which was found effective (44). The ability of many venoms to hemolyze red blood cells is well known (45-47). Rattlesnake venom however has little or no activity as a hemolysin or hemagglutinin whereas cottonmouth moccasin is very active (35). It is significant that in many of the studies moccasin or cobra venom is more potent than rattlesnake venom in disrupting membranes which agrees with our results.

TABLE III — PENETRATION OF VARIOUS C^{14} LABELLED COMPOUNDS INTO THE AXOPLASM OF CONTROL AND *AGKISTRODON P. PISCIVORUS* VENOM TREATED SQUID AXONS

All compounds were applied for one hour in a concentration of 4.5×10^{-3} M except for 1×10^{-3} M indoleacetic acid, 1.9×10^{-6} M dieldrin and 2.5×10^{-4} M diphenylhydantoin, cortisol and dehydroepiandrosterone sulfate. *Agkistrodon p. piscivorus* venom applied for 30 minutes in a concentration of 100 μg/ml blocked conduction. None of the other compounds had any effect on conduction during the 1 hour application. GABA = γ Amino butyric acid, DOPA = 3,4 dihydroxyphenylalamine, DOPA mine = 3,4 dihydroxyphenylethylamine. Data from Hoskin and Rosenberg (22).

COMPOUND	Per cent penetration	
	Control	Venom pretreated
Glucose	20	—
Mannitol	1	—
Sucrose	1	40
Glutamate	1	35
Glutamine	2	35
Aspartate	2	—
GABA	4	25
DOPA	2	35
DOPA mine	3	70
Serotonin	6	50
Indoleacetic acid	25	—
Acetylsalicylic acid	35	—
Diphenylhydantoin	60	—
Cortisol	100	—
Dieldrin	100	—
Dehydroepiandrosterone sulfate	4	—

The studies of the permeability properties of the squid giant axon were extended by measuring the penetration into the axoplasm of a wide variety of compounds (Table III). Except for glucose, penetration of the substances studied appears to depend largely on the extent of the non-polar, lipophilic character of the compound. This conclusion is based on experiments with squid axon, however it appears possible that cell membranes from widely different sources may have sufficient properties in common to give validity to such generalization. The glucose penetration appears to be metabolically mediated, indeed studies with specifically labeled glucose indicated the participation of the pentose phosphate pathway as a metabolic route in axonal membrane and associated cell wall material and partial or complete absence of the oxidative system in the axoplasm (22). Glutamate, glutamine, GABA, aspatate, DOPA, DOPA mine and serotonin, all of which penetrated poorly have two or more ionizable groups each, selected from carboxyl, amine and phenolic hydroxyl groups, and exist almost always in a charged form. In contrast aspirin, indolacetic acid and diphenylhydantoin which have only one ionizable group, and which would be in equilibrium with a significant amount of the dissociated lipophilic form, penetrated the untreated squid axon much more rapidly. Dieldrin is an extremely water-insoluble insecticide, and cortisol is a lipid-soluble steroid. In contrast dehydroepiandro-

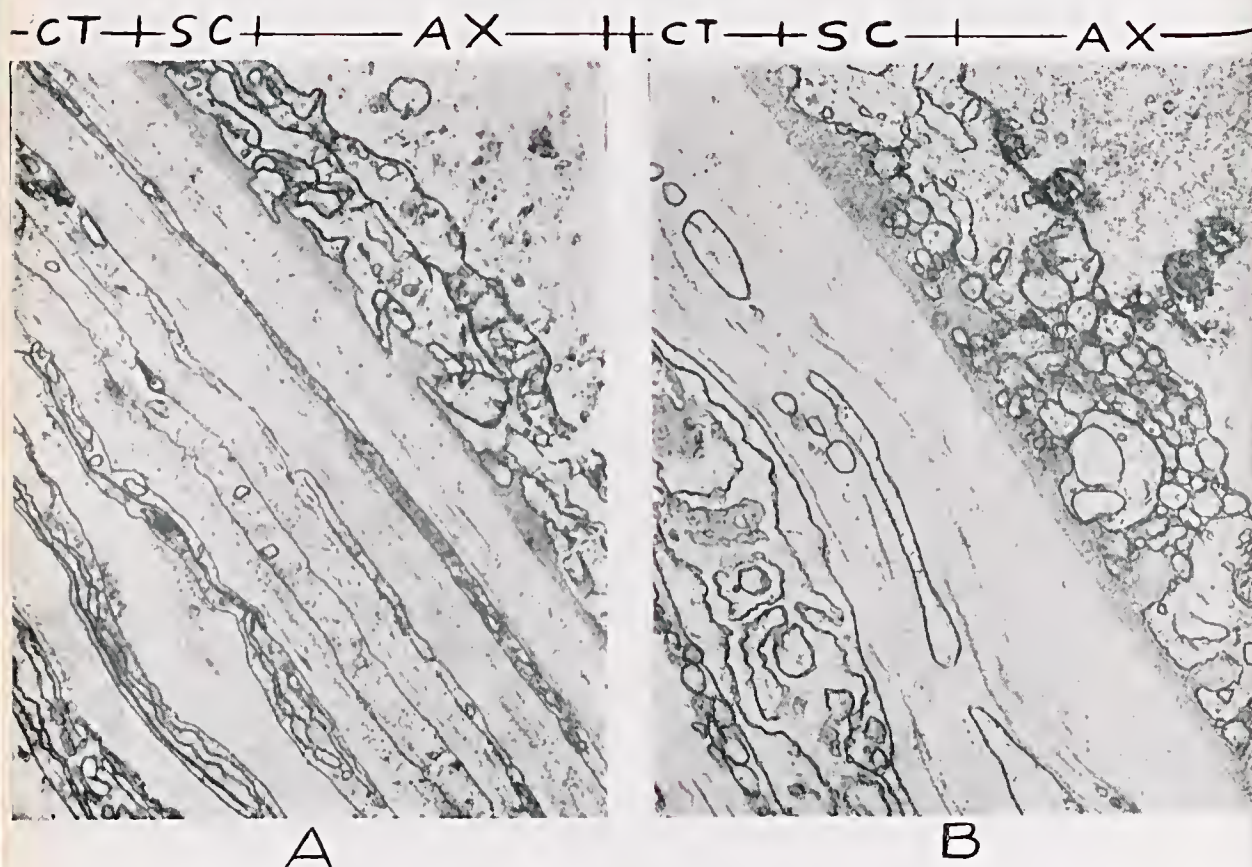



Fig. 11

sterone sulfate is a water-soluble completely ionized derivative of an otherwise water-insoluble steroid. The steroid and steroid sulfate thus constitute a striking example of the effect on permeability of introducing a highly polar completely dissociated substituent into a lipophilic molecule. Moccasin venom treatment seems to increase the penetration of many poorly penetrating compounds in a not too specific way.

The permeability barrier of the squid axon may be the axolemma, Schwann cell or connective tissue surrounding the axon. According to as yet unpublished observations of Dr. David Robertson with electron microscopy of venom pretreated axons, the venom produced marked alteration and disintegration of the Schwann cell; the axonal plasma membrane appeared to be not affected (Fig. 11). Villegas and Villegas (38) have shown that Schwann cells are traversed by relatively large channels, however their tortuous nature may still make penetration difficult. Higher concentrations of venom may block electrical activity by a gross disruption of the axonal membrane or its receptor areas. The venoms might also increase the concentration of curare and ACh which reaches the active sites by disrupting nonspecific binding sites with which charged molecules such as curare and ACh might be expected to interact (e.g. nucleic acid, chondroitin sulfate etc.).

Alteration of acetylcholine penetration into, and effects on venom-treated squid axons

An apparently surprising result in our studies with venom pretreated squid axons was the finding that low concentrations of physostigmine applied in combination with ACh did not enhance its action as would have been expected with a potent ChE inhibitor, but markedly antagonized its action. Curare in contrast did not antagonize the action of ACh (Table IV). Similar unexpected observations had previously been made on the vagus and lobster nerves (10, 48). Both of these findings are opposite to that observed at junctional sites where low concentrations of physostigmine enhance the action of ACh and curare antagonizes

TABLE IV — PENETRATION INTO AXOPLASM AND EFFECTS ON ACTION POTENTIAL (AP) OF VENOM TREATED SQUID GIANT AXONS BY C^{14} ACETYLCHOLINE (ACh) ALONE AND COMBINED WITH OTHER COMPOUNDS

Axons were exposed to 4.5×10^{-3} M ACh for one hour. All axons were pretreated with 25 µg/ml *Agkistrodon p. piscivorus* whieh had no effeet on the AP. Data taken from Hoskin and Rosenberg (19), and Rosenberg and Podleski (16).

COMPOUNDS	Per cent	
	Decrease AP	Penetration
ACh	90	4
ACh + choline 2.7×10^{-3} M	70	3
ACh + choline 5.4×10^{-3} M	40	—
ACh + eholine 2.7×10^{-2} M	20	1
ACh + neostigmine 1.7×10^{-2} M	30	1
ACh + physostigmine 5×10^{-4} M	30	1
ACh + physostigmine 5×10^{-3} M	40	1
ACh + eurare 1×10^{-4} M	90	—
ACh + curare 7×10^{-3} M	100	15

its action. Both choline and neostigmine. which even in high concentrations have no effect on conduction of the venom treated squid axon also markedly antagonize the effect of ACh on the action potential (Table IV).

Nachmansohn had suggested that there may be a competition between these compounds for the pathways of penetration in the structures surrounding the conducting membrane; i.e., external to the receptor areas of the membrane. We therefore undertook studies with radioactive compounds to see how well the effects on electrical activity correlated with the penetration of ACh. Choline, neostigmine and physostigmine decreased the penetration of ACh in venom treated axons from about 4 per cent to about 1 per cent which is the level of penetration observed in control axons not treated with venom (Fig. 10). In contrast curare did not decrease but actually increased the penetration of ACh (Table IV).

The results indicate that physostigmine, neostigmine and choline decrease the effects of ACh on conduction by competing with ACh for penetration pathways. The competition appears to be concentration dependent for choline, and choline seems to compete with ACh for penetration on a mole for mole basis since concentrations of choline about equal to that of ACh are needed to demonstrate antagonism. In contrast the antagonistic action of physostigmine is much more potent than that of choline since 5×10^{-4} M physostigmine decreased the penetration of ACh as much as 5×10^{-3} M agreeing with the observations on electrical activity. Since neostigmine, choline and the low concentration of physostigmine have no direct effects on conduction it would be difficult to assume that they are competing with ACh for a specific site in the membrane essential for electrical activity. This conclusion is further supported by the observation that it is no longer possible to demonstrate antagonism of ACh penetration by choline when the permeability barriers to ACh are no longer the limiting factor when the axons are pretreated with 100 μg/ml cottonmouth moccasin venom, a concentration which blocks electrical activity and markedly increases the penetration of ACh (Fig. 10).

These results confirm and extend the previous observations on the importance of structural barriers surrounding the axonal conducting membranes in determining effects of chemical compounds on electrical activity. ACh, choline, neostigmine and curare are all quaternary nitrogen derivatives while physostigmine at the pH used is partly in the charged form. They may all react with negatively charged groups in the reduced but still persisting barrier and may modify the effects observed when two of them are applied simultaneously. Apparently, however, they do not react with the same groups in the squid axon preparation since curare facilitates the passage of ACh while choline and neostigmine decrease its passage. In contrast curare markedly decreases the penetration of a quaternary nitrogen compound in skeletal muscle (49) emphasizing the variability of interaction between compounds which may be observed in different preparations.

It was of interest to investigate whether there was a degree of specificity about this competition phenomenon. The penetration of ACh was therefore tested in the presence of sucrose, a non charged pharmacologically indifferent compound. Sucrose had no significant effect on ACh penetration (23), indicating that charged molecules may more effectively compete with ACh for penetration sites than uncharged molecules. Our findings on the axon are due to the entirely different environment from that at the junction where for example neostigmine and physostigmine at low concentrations increase the action of ACh by the inhibition of AChE and neostigmine in addition by activating the receptor. The exact sites at

which these compounds antagonize the penetration of ACh is not known, but it must be kept in mind, that the Schwann cell surrounding the squid giant axon is about 2000 A thick compared to the 80 A thickness of the excitable membrane itself.

Externally added ACh decreases the action potential and depolarizes the rabbit vagus after removal of the sheath (48). However, the likelihood of there being a physiological significance to this effect was considered remote because neostigmine and other compounds which had no effect on conduction antagonized the action of ACh (50). As in the squid axon the conducting membrane in the vagus preparation is surrounded by a large layer of other structures, and thus it appears to us likely that an effect on penetration similar to that described here is the explanation for their findings.

Increased cholinesterase activity of intact cells caused by venoms

To determine whether snake venom increased permeability to ACh in preparations other than the squid giant axon it was necessary to use techniques not requiring the extrusion of the cells contents. It has been shown that the ratio of ChE activity in an intact and homogenized preparation, to which we shall refer to as the permeability constant (PC), serves as an index of the permeability barrier (51). If the ChE of the tissue were completely accessible to ACh, which is used as the substrate for measuring the ChE activity, then the PC would be 1, whereas complete inaccessibility would give a PC ratio of 0.

The PC ratios in control preparations of squid stellar nerve, eel electroplax, walking leg nerve of lobster, frog sartorius muscle and rabbit cerebral cortex indicated that the eel electroplax has the strongest barrier to ACh whereas the squid stellar nerve has the weakest (Table V). The squid stellar nerve is composed of the giant axon plus small nerve fibers. Since giant axons contain only 2-4% of the total ChE activity of the squid stellar nerve (52) these figures would not indicate the barrier present in the giant axon. Because of the relatively low enzyme activity of the giant axon it is not possible to measure small changes in its activity. The greatest fraction of ChE of the giant axon is located in the envelope. The surface area of the giant axon is much less than the total surface area of the small nerve fibers. Therefore, it is not surprising that the majority of the ChE is in the small fibers.

Agkistrodon p. piscivorus and to a lesser extent *Crotalus adamanteus* venom caused an increase in the ChE activity of intact axons of the squid stellar nerve, the fibers of the lobster walking leg nerve and the isolated eel electroplax (Table V). The greater effectiveness of cottonmouth than rattlesnake venom in increasing permeability agrees with previous findings in other systems that rattlesnake venom is less effective in its ability to disrupt membranes. Since the venoms used have no ChE activity themselves (20, 53, 54), their effects must be attributed to a greater accessibility of ChE in the intact preparations as previously discussed. In contrast to the complete ineffectiveness of Eastern diamondback rattlesnake venom on the squid giant axon (Table II and Fig. 10), it increased the permeability of the squid stellar nerve although less than the cottonmouth moccasin venom. The increased permeability caused by the venoms in lobster nerves agrees with the findings that cottonmouth venom decreases the concentration of ACh required to affect electrical activity (11).

TABLE V — EFFECT OF VENOMS ON PERMEABILITY OF VARIOUS PREPARATIONS AS JUDGED BY THE ABILITY OF 5 × 10^{-3} M ACETYLCHOLINE (ACh) TO PENETRATE AND ASSAY ALL OF THE AVAILABLE CHOLINESTERASE (ChE) IN INTACT BIOLOGICAL PREPARATIONS

The permeability constant (PC) is the ratio of ChE activity of intact and homogenized tissue. AP = *Agkistrodon piscivorus* venom; CA = *Crotalus adamanteus* venom. All ChE activities are expressed as μM ACh hyd/g/hr except for the electroplax results which are presented as μM ACh hyd/cell/hr (Average cell weight is 40 mg). Data taken from Rosenberg and Dettbarn (20, 25).

TISSUE	Venom	μg/ml	ChE		PC
			Homog.	Intact	
Squid Stellar Nerve	—	—	57	42	0.74
	AP	15	57	44	0.77
	AP	50	56	54	0.96
	CA	50	58	46	0.79
	CA	200	55	50	0.91
Eel Electroplax	—	—	18	3	0.17
	AP	50	20	5	0.25
	AP	400	29	10	0.34
	AP	1000	32	20	0.63
	CA	50	20	4	0.20
	CA	1000	32	9	0.28
Walking Leg Nerve of Lobster	—	—	925	337	0.36
	AP	100	900	442	0.49
	AP	1000	886	698	0.79
	CA	1000	900	475	0.53
Frog Sartorius Muscle	—	—	32	13	0.41
	AP	1000	32	27	0.84
Rabbit Cerebral Cortex	—	—	260	138	0.53
	AP	1000	285	152	0.53
	CA	1000	256	156	0.61

Permeability barriers are present in the intact rabbit cerebral cortex slices as is shown by the approximately doubling of ChE activity observed upon homogenization, but neither venom was able to significantly increase the accessibility of the enzyme to ACh in these slices. This is in agreement with earlier studies showing that *Naja naja* venom cannot hydrolyze phospholipids in brain slices (55). As we shall discuss in Section 8, splitting of phospholipids might be essential for increasing permeability. An interesting and unexpected finding was the demonstration in homogenized electroplax of strong permeability barriers. This was the only preparation in which the venoms increased the ChE activity even of homogenized tissue. The barriers in the homogenized tissue could be partially eliminated by longer periods of homogenization or by freezing and thawing of cells; however, the use of venoms appeared to yield the highest enzyme activity. In the homogenized electroplax both venoms were about equally effective in contrast to

the weaker effects of *Crotalus* venom in intact preparations. This may indicate an inability of rattlesnake venom components to penetrate to substrate sites in the membrane of intact preparations (see section 8).

Because, as discussed above, substrates such as ACh do not penetrate to all of the ChE it is difficult to determine the total enzyme activity in intact nerve fibers. In studies aimed at measuring ChE activity of a tissue following exposure to an "irreversible" organophosphate ChE inhibitor, it is essential to measure activity in intact preparations, since excess inhibition due to the presence of uncombined inhibitor may occur at the time of homogenization. Several procedures have been used in attempts to measure the total activity in the intact preparation. However none have been completely satisfactory (56, 57). Our results indicate that venoms may be useful in future studies where it will be essential to obtain some indication of the total ChE activity in intact preparations.

Use of venoms in testing for essentiality of cholinesterase in conduction

The ability of venoms to decrease permeability barriers in the squid axon and expose ChE to ACh (Fig. 10, Table V) offered the possibility of designing experiments to test for the essentiality in nerve conduction of ChE, the enzyme which hydrolyzes ACh. Organophosphates, such as diisopropyl phosphorofluoridate (DFP) or diethyl-p-nitrophenyl phosphate (Paraoxon) phosphorylate and thereby irreversibly inhibit ChE and block electrical activity (3-5). It is possible to reactivate the phosphorylated enzyme with certain nucleophilic compounds among which pyridine-2-aldoxime methiodide (PAM) is extremely potent and has a high degree of specificity for phosphorylated ChE (58). If block of conduction by organophosphates is specifically due to block of ChE and if ChE is essential for conduction as postulated by Nachmansohn (3-5) it might be possible by using PAM to specifically restore conduction. Experiments of this type are extremely difficult to carry out however, because PAM, like ACh and curare being a lipid insoluble quaternary nitrogen compound cannot penetrate the conducting membranes of nerve and muscle. In the lobster axon where the permeability barrier is less as evidenced by the ability of ACh to directly block conduction (10, 11), it was possible to overcome conduction block by Paraoxon with PAM (59). It was therefore worthwhile to test on the venom treated squid axon, where the permeability barriers are reduced and the active sites of ChE exposed, whether PAM could restore electrical activity which had been blocked by DFP or Paraoxon.

It was found that in axons not venom pretreated the block of conduction by Paraoxon, in the concentrations used and the time of exposure applied, was reversible whereas in axons treated with 25 $\mu g/ml$ cottonmouth moccasin venom the block was irreversible (25). At the time of reversible block of conduction ChE activity is still present and in no case was conduction found in the absence of enzyme activity (3-5, 60, 61). This indicates that Paraoxon may be reacting with some component of the membrane other than ChE, possibly with the active site of the receptor, to cause reversible block. The irreversible conduction block by Paraoxon after venom may be either because of the greater exposure of ChE to Paraoxon or because venom pretreatment inactivates the enzyme which hydrolyzes organophosphates. It was found that the squid axon has considerable amounts of this enzymatic activity, both in the envelope and in the axoplasm (62). Conduction in 14 out of 16 venom treated axons was irreversibly blocked by

Paraoxon, whereas in axons exposed to PAM following Paraoxon the block of conduction by Paraoxon was irreversible in only 6 of 17 axons (25). An actual experiment with Paraoxon and PAM is shown in Fig. 12. PAM also restored electrical activity which had been blocked by DFP (25). These experiments confirmed the inseparable association between electrical and ChE activity.

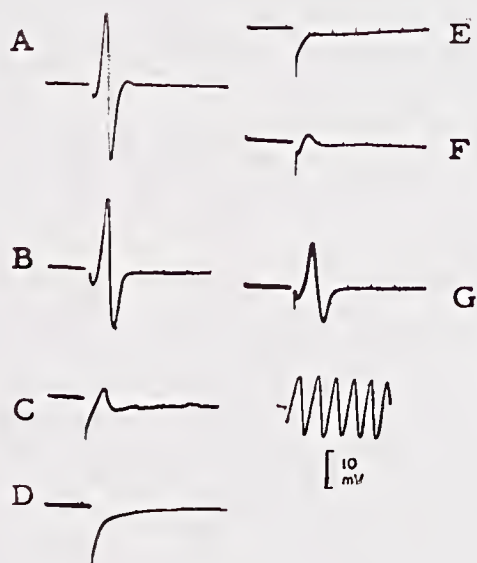



Fig. 12

MECHANISM OF VENOM ACTION ON THE SQUID GIANT AXON

In addition to non-enzymic components venoms contain many enzymes including phospholipase A, hyaluronidase, proteolytic enzymes, l-amino acid oxidase, phosphodiesterase, etc. (45, 46, 63-66). Venoms of the ELAPIDAE such as *Naja naja* venom have ChE activity (53, 54). It is obviously of great interest to determine the component (or components) of the venoms responsible for increasing the permeability of the squid giant axon.

The effects of various venom fractions, enzymes and phospholipids are shown in Table VI. Those compounds which blocked the action potential did so irreversibly. The concentrations of these agents used as pretreatment had no effects on the action potential. The effect of curare following phospholipase D was irreversible whereas all other curare effects were reversible.

A non dialyzable fraction of *Naja naja* venom rich in phospholipase A (17, 67) was more potent than *Naja naja* venom (Table II) both in its direct effects on the action potential and as a pretreatment agent (Table VI). ACh also affected conduction after the phospholipase rich fraction. In contrast a dialyzable fraction of cobra venom poor in phospholipase A (17, 67), required relatively high concentrations to block conduction, and following pretreatment of the axon with it curare was completely inactive (17). The comparative phospholipase A activities of the rich and poor fractions shown in Fig. 13, indicate that the rich fraction is at least 200 times as active as the poor fraction.

TABLE VI — CONCENTRATIONS OF SEVERAL VENOM FRACTIONS, ENZYMES AND PHOSPHOLIPIDS USED AS PRETREATMENTS TO RENDER THE SQUID AXON SENSITIVE TO CURARE, AND CONCENTRATIONS REQUIRED TO BLOCK THE CONDUCTED ACTION POTENTIAL (AP)

Compounds were applied for about 30 minutes. > indicates that at the concentration shown no effect on AP was observed. +++, ++, and — indicate respectively a 80-100%, 50-80% and 0-20% decrease in the AP produced in 30 minutes or less by 1.4×10^{-3} M curare after pretreatment of the axon. OH— and H+ indicates that venom solution was boiled for 15 minutes at pH 8.5 or 5.5 after which the solution was cooled, pH readjusted and then tested. Data taken from Rosenberg and Podleski (15) and Rosenberg and Ng (17).

COMPOUND		µg/ml		Curare effect
		AP block	Pretreat	
Phospholipase A rich fraction		20	2	+++
Phospholipase A poor fraction		200	100	—
Agkistrodon p. piscivorus	OH—	> 1000	200	—
Agkistrodon p. piscivorus	H+	200	50	+++
Naja naja	OH—	> 400	200	—
Naja naja	H+	100	50	++
Phospholipase C		> 500	500	—
Phospholipase D		> 100	10	++
L-Amino acid oxidase		> 100	100	—
Hyaluronidase		> 100	100	—
Cobroxin		500	200	—
Cobra venom Neurotoxin		> 250	250	—
Lysolecithin		500	100	—
Egg lecithin		> 1000		
Beef heart lecithin		> 1000		

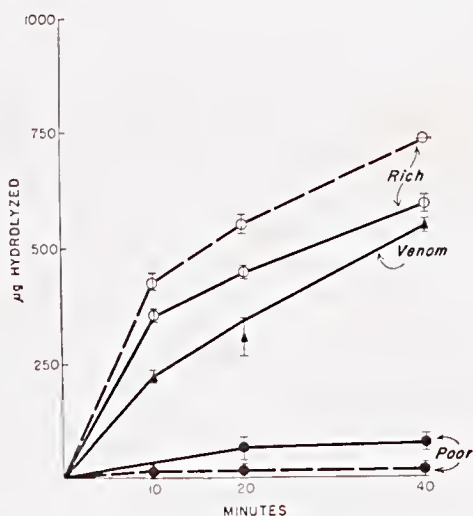



Fig. 13

Phospholipase A in venoms is resistant to boiling at acid pH whereas it is destroyed by boiling at an alkaline pH (68-70). The other enzymes known to be present in venom are destroyed by boiling at acid or alkaline pH. Some

investigators therefore use acid heated venom solutions as their source of phospholipase A. As seen in Table VI alcaline heated solutions of *A. piscivorus* and *Naja naja* did not render curare active while acid heated solutions were effective. An acid heated solution of cobra venom has also been found to depolarize and block conduction in lobster axons (30). The comparative phospholipase activities of the acid and alkaline heated solutions are shown in Fig. 14. These results also indicated that phospholipase A is responsible for decreasing the permeability barrier in the squid giant axon.

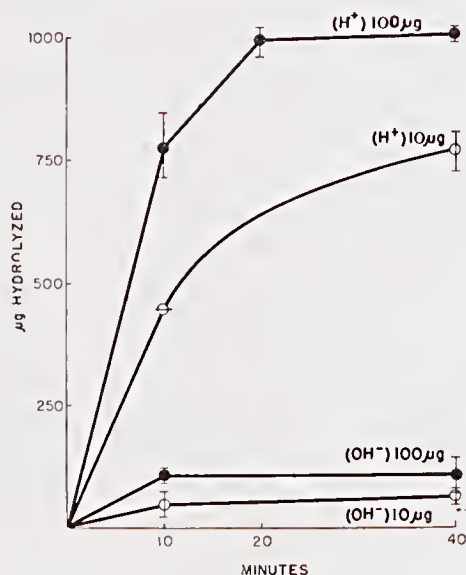



Fig. 14

Neither 1-amino acid oxidase nor hyaluronidase, both of which are present in snake venoms, had any effect on the squid axon (Table VI). A preparation of phosphodiesterase from venom appeared to be highly potent, however we found it to contain considerable phospholipase A activity (17). Two different preparations of neurotoxin from *N. naja* venom (Cobroxin and cobra venom neurotoxin), although highly toxic to animals were also inactive on the squid axon. No relationship has been found between venom toxicity and any of the enzymatic fractions in venom (64, 71-74). As noted in Table VI three phospholipids were inactive as also were phosphatidylethanolamine, phosphatidylserine, L-α-cephalin and L-α-lecithin. Phospholipases C and D are not present in snake venoms and we shall discuss their actions later.

The results shown in Table VI indicate that phospholipase A of venoms is responsible for their ability to increase the permeability of the squid axon to normally impenetrable lipid-insoluble compounds. Also in support of this supposition is the finding that *Notechis scutatus* a venom highly potent in our studies (Table II) also contains high concentrations of phospholipase A (75). In contrast *Centruroides sculpturatus* (scorpion) venom which contains no phospholipase A (76) was inactive in our test system (Table II), although it produced repetitive firing of the squid axon (24). As shown in Fig. 15 phospholipase A hydrolyzes a fatty acid ester from the β position of certain glycerophosphatides yielding the

	X_1	X_2	X_3
Phosphatidylcholine (lecithin)	O ‖ C—R	$(CH_2)_2$	$(CH_3)_3$
Phosphatidylethanolamine	O ‖ C—R	$(CH_2)_2$	H_3
Phosphatidylserine	O ‖ C—R	CH_2—CH (COO(-))	H_3
Phosphatidalcholine (choline plasmalogen)	CH=CH—R	$(CH_2)_2$	$(CH_3)_3$

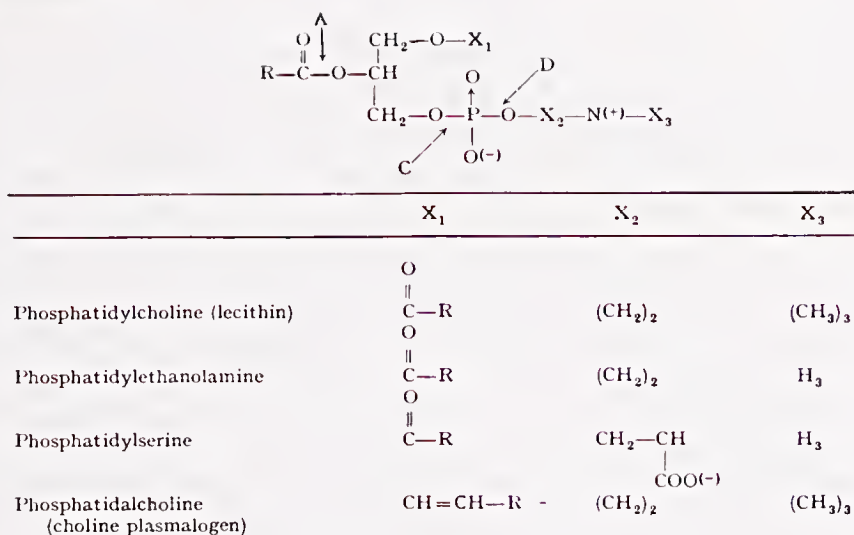



Fig. 15

corresponding lysophosphatide. Previous studies by many workers have shown that phospholipase A is capable of disrupting membranes (33, 34, 69, 77, 78). The source of phospholipase A in most of these studies was acid heated solutions of venom. Since we observed such a great variability in venom potency (Table II) we decided to compare the phospholipase A activities of certain venoms. In most of the measurements of phospholipase A activities we used egg or beef heart lecithin rather than purified substrates because hydrolysis was slower and incomplete with purified substrates (17). Snake venom phospholipase A has been reported to hydrolyze egg yolk and serum phospholipids at a rate 10 to 20 fold greater than that obtained with purified ovolecithin preparations, and it was suggested that this may be due to the specific nature of the combination of phospholipid and protein in the crude substrate preparation (79). The sample of beef heart lecithin used contained about 60% phosphatidalcholine (choline plasmalogen) and 40% phosphatidylcholine (lecithin). It was reported by Gottfried and Rapport (80) that cobra venom hydrolyzes egg lecithin at only a slightly faster rate than beef lecithin whereas Western diamondback rattlesnake venom hydrolyzes egg lecithin considerably better than beef lecithin. In addition when they obtained purified plasmalogen from beef lecithin the difference in hydrolysis of lecithin and plasmalogen by rattlesnake venom was even more marked. Since Eastern diamondback rattlesnake venom did not render curare active whereas cobra and cottonmouth moccasin venom did it appeared that an action of the venom upon plasmalogen might be responsible for their ability to render curare active. As seen in Fig. 16 we found that cottonmouth venom hydrolyzed the two substrates at similar rates, whereas rattlesnake venom is somewhat more effective in hydrolyzing egg than beef lecithin. Quantitatively however this difference in reaction rate does not appear sufficient to account for the marked differences in potency of these two venoms on the squid axon. When the hydrolysis of beef lecithin by various venoms was compared it was found that two of the venoms most effective in rendering curare active also hydrolyzed beef lecithin at the highest rates, but hydrolysis by the other three venoms did not correlate with their ability to render curare active (Fig. 17). It will be necessary to actually

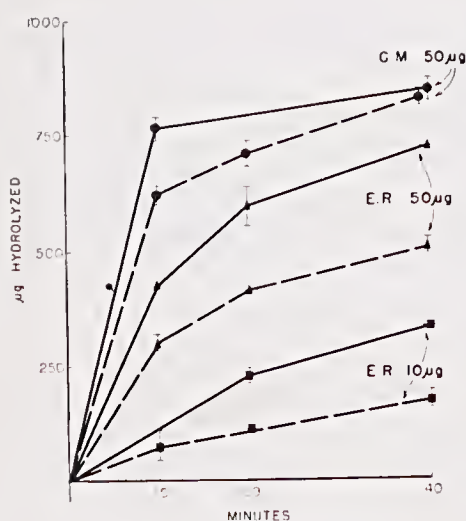



Fig. 16

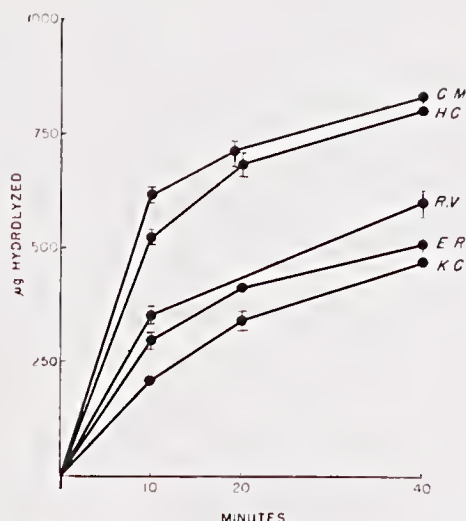



Fig. 17

measure the phospholipids in the squid axon as well as the changes produced by cottonmouth and rattlesnake venoms to see if there is a correlation between splitting of any particular phospholipid and potency of the venoms. The nervous system of course contains many phospholipids in addition to lecithin and choline plasmalogen (phosphatidalcholine); for example, phosphatidylethanolamine, phosphatidylserine, phosphatidalethanolamine, phosphatidalserine, inositol phosphatides and sphingomyelins (81-87). The concentration of phosphatidalcholine is actually much less than that of phosphatidalethanolamine (87, 88). Lecithin is not even a comparent of the myelin lipids whereas plasmalogens, inositol phosphatides and sphingomyelins are (84, 89, 90). Quantitatively plasmalogens form one of the largest part of the phosphatides found in white matter (83, 91).

In addition to the possibility that the phospholipase A in cottonmouth and rattlesnake venoms have different specificities toward phospholipid substrates it is also possible that there are isozymes of phospholipase A which have differences in their molecular structure, which may explain the marked differences in their potencies. It has been reported that there are differences in the electrophoretic migration of the phospholipase A fractions from different venoms (92, 93). It has also been pointed out that it can no longer be assumed that a single enzyme in each venom is responsible for the hydrolysis of all phospholipid substrates (80). In certain venoms there have been found more than one phospholipase A, each with different physical properties (75, 94), and different specific activities when tested on various phospholipids. It is of special pertinence to our data that the phospholipase A from Ringhals and *Naja naja* venom are able to split phospholipids in certain membranes whereas the phospholipase A from *V. palestinae* is unable to do so (55, 95). Similar differences could be responsible for the marked difference in potency between rattlesnake and cottonmouth venom.

In addition to the possibility that the phospholipases from rattlesnake and cottonmouth venoms have different substrate specificities or different molecule structure there is also the possibility that there is another factor in the active venoms which prepares the membrane for attack by venom phospholipase. Such a factor, the direct lytic factor (DLF) has been isolated from cobra and bee

venoms, but is not present in certain viper venoms (73, 93, 96-98). For example washed erythrocytes are resistant to the action of phospholipase A obtained from various venoms, whereas the addition of DLF, which itself has no phospholipase A activity renders the red cell phospholipids susceptible to attack by phospholipase A of these venoms (96). DLF might allow venom phospholipase A to slipt the phospholipids in the intact squid axon. If this factor were absent in rattlesnake but present in cottonmouth venoms this could explain our results.

Assuming that phospholipase A is the active component of the venoms it is of interest to know whether the venom effects are due to a direct action of phospholipase A on phospholipids or due to the liberation, by the action of phospholipase A on phosphatides, of lysophosphatides in the membrane. For example lysolecithin a compound with detergent properties is formed by the hydrolysis of the fatty acid ester at the β position of lecithin by phospholipase A (Fig. 15). It is thought that indirect hemolysis of red blood cells by venoms is due to the action of the lysophosphatides formed from the action of phospholipase A on the membranal phospholipids (99). Both phospholipase A and lysolecithin cause demyelinating changes in the central nervous system (33), while lysolecithin and venom cause a release of glutamic-oxaloacetic transaminase from whole cell preparations (43). It was also suggested that block of conduction in lobster nerves by heated venom solutions may be due to the formation of lysophosphatides (30). Other investigators (77, 78, 100, 101), for various reasons, think that the effects they have observed are due to a direct action of phospholipase A and not due to a liberation of lysophosphatides. For example, in two of these studies (78, 100), phospholipase C had a similar effect as heated venom solutions which was taken as evidence that a lysophosphatide is not important in the action of phospholipase A. The action of phospholipase C on phosphatides does not give rise to lysophosphatides (Fig. 15). In our studies however phospholipase C was inactive (Table VI).

In attempting to decide whether phospholipase A is acting directly or whether lysophosphatides are responsible for increasing permeability an observation made on squid giant axons after removal of other fibers is of relevance. After having found that ACh was inactive on conduction and unable to penetrate both on cleaned and less carefully dissected axons (see section 2 and 3) we have for the sake of convenience used giant axons with only partial removal of surrounding fibers. The apparently surprising observation was later made that cottonmouth venom is practically inert on axons carefully dissected, that is, with all small nerve fibers and as much connective tissue as possible removed (15). Following application of even high concentrations of cottonmouth venom on finely dissected axons, curare and ACh were inactive and penetrated no better than in axons not pretreated with venom (23). These results are in marked contrast to effects of the detergent cetyltrimethylammonium bromide which is known to directly disrupt membranes, and which was even more potent on the finely dissected axon than on the preparation without removal of small fibers (23). A likely explanation for the observation with venom on cleaned axons is that the venom is acting through the formation in or near the membrane of a secondary product which in turn affects electrical activity by disrupting barriers either within or external to the membrane. In the closely dissected axon there would be less substrate on which phospholipase A could act and therefore less lysophosphatides formed. The lyso compounds may also increase the activity of phospholipase A (102). Attempts were made to demonstrate the production by cottonmouth venom of a compound with potent effects on electrical activity. The venom was incubated

with squid axons and with egg and beef lecithin, but no compound was obtained from the incubation mixture with potent effects on electrical activity. These negative results obviously do not exclude the possibility that such compounds may be formed since adding a lysophosphatide externally may not give results equivalent to its formation within the preparation.

Although phospholipase C and D are not present in venoms, the effects of these two compounds on the squid axon are interesting. While neither of them had any direct effects on the action potential, phospholipase D even in relatively low amounts rendered curare active (Table VI). The block of conduction by curare after phospholipase D could however not be reversed. Hydrolysis of phosphatides by phospholipase D removes a cationic nitrogen (Fig. 15). One may speculate that the cationic nitrogens of curare may be attracted to the negatively charged phosphates. The curare molecules may pass from one phosphate to another and finally penetrate to the active sites of the neuronal membrane and affect electrical activity. The concentration gradient of curare and its binding might be such that with washing it would be difficult to remove enough of the curare to reverse its effects on electrical activity. Hydrolysis of phosphatides by phospholipase C removes the phosphate grouping as well as the cationic nitrogen leaving an uncharged diglyceride which would not be expected to bind curare.

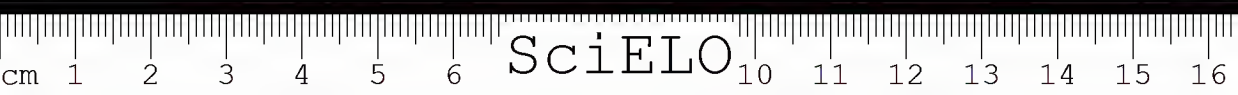
Summary

It has been possible with the aid of venoms to analyze some of the effects which the structure and organization of biological membranes and their environment have on the physiological functioning of the acetylcholine (ACh) system and on the action of compounds when applied externally to biological tissues.

1) Lipid soluble tertiary nitrogen containing compounds such as atropine and physostigmine which are known to interact with the ACh system at junctions also block conduction in the squid giant axon, indicating the presence of the ACh system in this preparation. In contrast lipid insoluble quaternary nitrogen containing compounds such as ACh and curare do not affect conduction of the axon.

2) ACh and curare reversibly block conduction after pretreatment of the squid axon with concentrations of certain snake venoms which by themselves have no affect on conduction. Of 21 venoms tested *Agkistrodon p. piscivorus* (cottonmouth moccasin) venom was the most effective in rendering the axon sensitive to curare whereas *Crotalus adamanteus* (Eastern diamondback rattlesnake) venom was completely inactive.

3) ACh and curare do not penetrate through the structural barrier surrounding the excitable membrane of the giant axon in contrast to lipid soluble tertiary nitrogen derivatives which can penetrate. Cottonmouth moccasin venom markedly increased the permeability of this structural barrier as evidenced by increased penetration of radioactive ACh, curare and choline. In contrast Eastern diamondback rattlesnake venom did not increase permeability. Cottonmouth venom also increased the permeability of the barrier to various sugars, amines and amino acids. The barrier is formed presumably to a large extent by the Schwann cell. Preliminary electron microscopic studies indicate that fragmentation of the Schwann cell surrounding the giant axon by venom may be responsible for the increased permeability.

4) Choline, neostigmine and physostigmine when applied in combination with ACh to venom treated axons markedly decreased the effect of ACh. This action was shown to be due to a competition for sites of penetration, thereby allowing less ACh to reach the excitable membrane.

5) In addition to increasing penetration of ACh in the squid giant axon, cottonmouth venom also increased its penetration in the squid stellar nerve, isolated single eel electroplax, walking leg nerve of lobster and frog sartorius muscle.

6) It was possible to demonstrate the essentiality of cholinesterase for conduction on the venom treated squid giant axon. The organophosphate cholinesterase inhibitor Paraoxon caused a block of axonal conduction which could not be reversed by washing with sea water but which was readily reversed by pyridine aldoxime methiodide, a specific reactivator of organophosphate inhibited cholinesterase.

7) It appears that phospholipase A is at least one of the components responsible for the action of venoms on the squid giant axon.

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Legends

Fig. 1 — Schematic presentation of the elementary process controlling membrane permeability during electrical activity and integration of ACh into the metabolic pathways. The role of ACh in the permeability cycle may be pictured as follows: 1) In resting condition ACh is bound to a storage protein (S). The membrane is polarized. 2) ACh is released by current flow; the free ester combines with the receptor protein (R). A conformational change of the receptor (symbolized by dotted line) leads to a shift of charge; this process increases permeability; it is the trigger action by which the ionic concentration gradient becomes effective. 3) The ester receptor complex is in dynamic equilibrium with free ester and receptor. The free ester is attacked by ACh-esterase (E). 4) The hydrolysis of the ester permits the receptor to return to its original condition. The permeability barrier is reestablished and the membrane is repolarized (From Nachmansohn, 3).

Fig. 2 — Cross section of the giant axon of squid (above) compared to that of rabbit sciatic nerve (below) at the same magnification (From Young, 13).

Fig. 3 — Effect of 1.4×10^{-3} M curare on squid axon rinsed in normal sea water (W) for 10 min. (left) and for 95 min. (right) after the application, per ml, of 10 μg of cobra venom (CV) plus 20 μg of cetyltrimethylammonium chloride (CTA) (From Rosenberg and Ehrenpreis, 14).

Fig. 4 — Effect of d-tubocurarine chloride (curare) on the giant axon of squid following pretreatment with 15 μg/ml cottonmouth moccasin venom. S. W. indicates return to sea water (From Rosenberg and Podleski, 15).

Fig. 5 — Effect of curare on the resting and action potential of the squid giant axon following exposure to cottonmouth venom. A, control; B, after exposure to 15 μg/ml venom for 15 min.; C, 15 min. after return to sea water (stimulus voltage remained constant for this period); D, E, 4 and 8 min. after exposure to 1.4 mM curare; F, 22 min. after return to sea water (From Rosenberg and Podleski, 16).

Fig. 6 — Effect of 67 μg/ml of hooded cobra venom on the resting potential (.—.) and action potential (o—o) of the giant axon of squid (From Rosenberg and Podleski, 15).

Fig. 7 — Effect of 50 μg/ml of cottonmouth moccasin venom on the resting potential (.—.) and action potential (o—o) of the giant axon of squid (From Rosenberg and Podleski, 15).
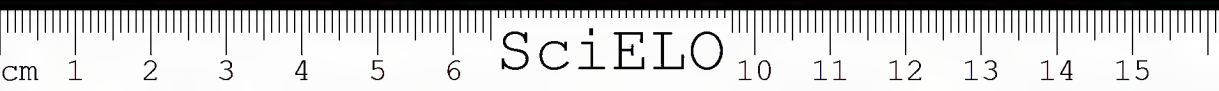
Fig. 8 — Effect of acetylcholine (ACh) on the electrical activity of the giant axon of squid following exposure to 15 μg/ml of cottonmouth moccasin venom. S. W. indicates return to sea water (From Rosenberg and Podleski, 15).

Fig. 9 — Effect of acethylcholine on the resting and action potential of the squid giant axon following exposure to cottonmouth venom. A, control; B, after exposure to 15 μg/ml venom for 30 min.; C, 15 min. after return to sea water; D, E, 15 and 25 min. after exposure to 4.4×10^{-3} M ACh; F, 30 min. after return to sea water (From Rosenberg and Podleski, 16).

Fig. 10 — Penetration of radioactively (C^{14}) labeled ACh, choline, dimethylcurare and trimethylamine into the axoplasm of squid giant axon with and without exposure to cottonmouth moccasin venom. The percentage indicates the radioactivity of the axoplasm compared to that in the outside fluid. The figures below the columns indicate the μg of venom/ml. In contrast to the moccasin venom, that of the Eastern diamondback rattlesnake (R. S.), even in 1000 μg/ml had no significant effect on the penetration of dimethylcurare. Trimethylamine readily penetrates with and without exposure to venom (according to data of Rosenberg and Hoskin, 18).

Fig. 11 — Electron micrographs of squid giant axons A, control and B, after treatment with 100 μg/ml *Naja naja* venom. $KMnO_4$-Pb stains used. Magnification of A = 17,000, B = 25,000. AX = axoplasm; SC = Schwann cell; CT = connective tissue. The axolemma of the squid giant axon is between Schwann cell and axoplasm. Vesiculation and fragmentation of venom treated Schwann cell is seen. (By courtesy of Dr. David Robertson unpublished observations).

Fig. 12 — Restoration by pyridine-2-aldoxime methiodide (PAM) of electrical activity blocked by Paraoxon in venom treated squid giant axon. A, control; B, after exposure to 25 μg/ml cottonmouth moccasin venom for 30 min.; C, D after exposure to 0.01 M Paraoxon for 2 and 5 min.; E, 30 min. after return to sea water; F, G, after exposure to 0.05 M PAM for 5 and 20 min. Time signal is 750 cycles per second (From Rosenberg and Dettbarn, 25).

Fig. 13 — Phospholipase A activities of 5 μg of phospholipase A rich (Rich) and 100 μg of phospholipase A poor (Poor) fractions of *Naja naja* venom and activity of 5 μg of *Naja naja* venom (Venom). 1 mg of egg (———) or beef (— — —) lecithin was the substrate. Results are shown as means ± S. E. of the mean (From Rosenberg and Ng, 17).

Fig. 14 — Phospholipase A activity of cottonmouth moccasin venom heated at pH 5.5 (H+) and pH 8.5 (OH−). Results are shown as means ± S. E. of the mean. Egg lecithin (1 mg) was the suhstrate (From Rosenberg and Ng, 17).

Fig. 15 — Structure of several glycerophosphatides. A, C and D indicate the points of hydrolysis by phospholipase A, C and D, respectively. R = hydrocarbon chain (From Rosenberg and Ng, 17).

Fig. 16 — Phospholipase A activity of cottonmouth moccasin (C.M.) and Eastern diamondback rattlesnake (E.R.) venom. 1 mg of egg (———) or beef (— — —) lecithin was the substrate. Results are shown as means ± S. E. of the mean (From Rosenberg and Ng, 17).

Fig. 17 — Phospholipase A activities of 50 μg of cottonmouth moccasin (C.M.), hooded cobra (H.C.), Russells viper (R.V.), Eastern diamondback rattlesnake (E.R.) and king cobra (K.C.) venoms. 1 mg of beef lecithin was used as substrate. Results are shown as means ± S. E. of the mean (From Rosenberg and Ng, 17).

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Discussion

C. Y. Lee: "I would like to ask your comment on the paper by Feng & Hsieh who demonstrated that the nerve action potential is unaffected after complete inactivation of the acetylcholinesterase by TEPP."

P. Rosenberg: "The difficulty in studies attempting to correlate cholinesterase activity and electrical activity is the method used to measure enzyme activity. The tissue cannot be homogenized since excess inhibition may occur at the time of homogenization due to presence of excess uncombined inhibitor. If one uses intact tissue with acetylcholine as substrate then one is only measuring the readily available or "external" cholinesterase since acetylcholine cannot penetrate to all of the enzyme. We have discussed all of these problems in a publication a few years ago in "Biochemical Pharmacology". Feng and Hsieh did not take into consideration all of the sources of error in this type of study."

F. Kornalík: "Have you got all the snake venoms from one source only?"

P. Rosenberg: "Most of the venoms were obtained from the Ross Allen Reptile Institute, Silver Springs, Florida, except for some of the Australian venoms which were obtained from Light & Co., England, and a few which were kind gifts of investigators from various parts of the U.S.A. and which are acknowledged in my publications."

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54. SEROTONIN AND RELATED TRYPTAMINE DERIVATIVES IN SNAKE VENOMS

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Introduction

Certain biogenic amines such as acetylcholine, serotonin and histamine often are found in venoms in very large amounts compared with other natural sources (Table I). Since these substances are known to produce pain when applied to blister areas it has been suggested that they play a defensive role rather than contribute in any important degree to the toxicity of the venom (1, 2).

TABLE I — SOME EXCEPTIONALLY HIGH LEVELS FOR CERTAIN OF THE LOW MOLECULAR WEIGHT COMPONENTS OF VENOMS

Species	Common name	Value Acetylcholine	Reference
Vespa crabro	(hornet)	10-100 mg/g dry venom	(11)
		10-50 mg/g dry venom	(12)
Zygaena spp.	(moths)	1.6-60 mg/g (Accessory sex glands and ducts. Organs of defense?)	(13)
Dendroaspis (3 species)	(mambas)	7-30 mg/g dry venom	(3)
		5-Hydroxytryptamine (Serotonin)	
Leiurus quinquestriatus	(scorpion)	up to 4 mg/g dry venom	(14)
Phoneutria fera	(Brazilian spider)	1.5-2.7 mg/g dry venom	(15)
Vespa crabro	(hornet)	up to 19/mg/g dry venom sacs	(12)
Synoeca surinama	(wasp)	13 mg/g dry sting apparatus	(15)
Rana pipiens	(frog)	0.3-1.0 mg/g wet skin	(2)
Other AMPHIBIA (many species)		up to 4.5 mg/g dry skin	(16)
		Histamine	
Apis mellifera	(honey bee)	10 mg/g dry venom	(17)
Vespa vulgaris	(wasp)	10 mg/g dry venom sacs	(18)
Vespa crabro	(hornet)	14-30 mg/g dry venom sacs	(12)

Supported by Grant NB-00623 from the Institute of Neurological Diseases and Blindness, USPHS.

Until recently, little attention has been paid to the possible occurrence of such substances in snake venoms. Acetylcholine has now been found in large amounts in the venoms of three species of *Dendroaspis* (3). However, the occurrence of serotonin and catecholamines in snake venoms is in dispute. Zarafonetis and Kalas (4) report their presence in venoms of *Crotalus atrox, C. adamanteus* and *Agkistrodon piscivorus*, but Anton and Gennaro (5) failed to find serotonin or norepinephrine in the venoms of the two latter species.

In a further study of this problem the venoms of 20 species of elapid, viperid and crotalid snakes have been examined by bioassay, spectrofluorometry, and chromatography for the possible presence of serotonin and related indolealkylamines. The results will be presented in this paper.

Materials and methods

The species of snakes whose venoms have been studied are listed in Table II. Venoms from seven species of Brazilian *Bothrops* were obtained from the Butantan Institute through the kindness of Dr. Hoge. Venom samples of the remaining species were purchased from the Miami (Florida) Serpentarium. In most instances only one pooled sample of venom from a given species was studied.

Three methods were employed to detect the presence of serotonin or related substances. However, some venoms were examined by only one or two of the following methods:

1) *Bioassay*. The isolated heart of the clam, *Mercenaria (Venus) mercenaria*, which detects small amounts of serotonin and related compounds (6), was used according to the method described by Welsh and Twarog (7).

2) *Spectrofluorometry*. Venoms were extracted according to the method of Bogdanski *et al.* (8) and fluorescence and excitation spectra were recorded with a Farrand spectrofluorometer in the presence of 3*N* HCl.

3) *Chromatography*. Venoms were extracted with 50 per cent acetone or 70 per cent ethanol and the concentrate subjected to paper and silica gel chromatography. Several developing systems suitable for separation indole compounds were used, as well as several standard methods for visualizing such substances.

Results

The results are summarized in Table II. Certain details concerning procedures, and the significance of the results obtained with each method of assay follow:

BIOASSAY — No bioassays were done with venoms of the Brazilian species of *Bothrops*. Whole venoms of all other species were tested on one or more isolated *Mercenaria* hearts. After dissolving in distilled water, from 0.01 mg to 0.25 mg of venom per ml of perfusion fluid was added to the bath. Tests were made before and after treatment of the hearts with benzoquinonium (Mytolon) in order to block the possible inhibitor action of acetylcholine which is present in some venoms (e.g. *Dendroaspis* in very large amounts (3)).

TABLE II — SUMMARY OF RESULTS OF THE THREE METHODS FOR DETECTING SEROTONIN

SPECIES	5-HT (Bioassay)	5-HT (Spectrofluorometry)	Indole-reacting spots (Chromatography)
ELAPIDAE			
ELAPINAE			
Bungarus fasciatus	+?	None detected	None
Hemachatus haemachatus	+?	None detected	1
Naja naja	—	None detected	1
Pseudechis mortonensis	+?	None detected	None
DENDROASPINAE			
Dendroaspis polylepis	—	None detected	1
Dendroaspis angusticeps	—	Not run	?
VIPERIDAE			
VIPERINAE			
Vipera russellii	+?	1.9 µg/g	2
Bitis gabonica	+	30 µg/g	4
CROTALINAE			
Crotalus horridus	—	None detected	2 or 3
Sistrurus m. barbouri	+	6.3 µg/g	4
Agkistrodon contortrix	+	Not run	4
Agkistrodon piscivorus	+	1.7 µg/g	5
Bothrops atrox	+	None detected	3
Bothrops (7 Brazilian species)	Not run	None detected	Not run

Serotonin and certain related compounds cause an increase in amplitude and frequency of beat of the *Mercenaria* heart, with little increase in tonus until relatively high concentrations are reached. The catecholamines and histamine also have an excitor action but the *Mercenaria* heart is far less sensitive to these than to serotonin. Also, they cause an increase in tonus resulting in a marked rise in baseline. Methysergide (UML 491) is an effective blocking agent for serotonin on the *Mercenaria* heart but not for catecholamines and histamine (9). When a venom was seen to excite the heart it was tested again after the addition of methysergide to the bath.

A sample recording of the actions of *Bitis gabonica* and *Sistrurus m. barbouri* venoms before and after methysergide is seen in Fig. 1. The exciter action of *Bitis* venom is completely abolished by methysergide and that of *Sistrurus* venom is much reduced. This suggests that serotonin or a close relative is present in these venoms. The failure of methysergide to block completely the exciter action of *Sistrurus* venom, at the given dose level, might be due to the presence of a catecholamine or histamine in this venom.
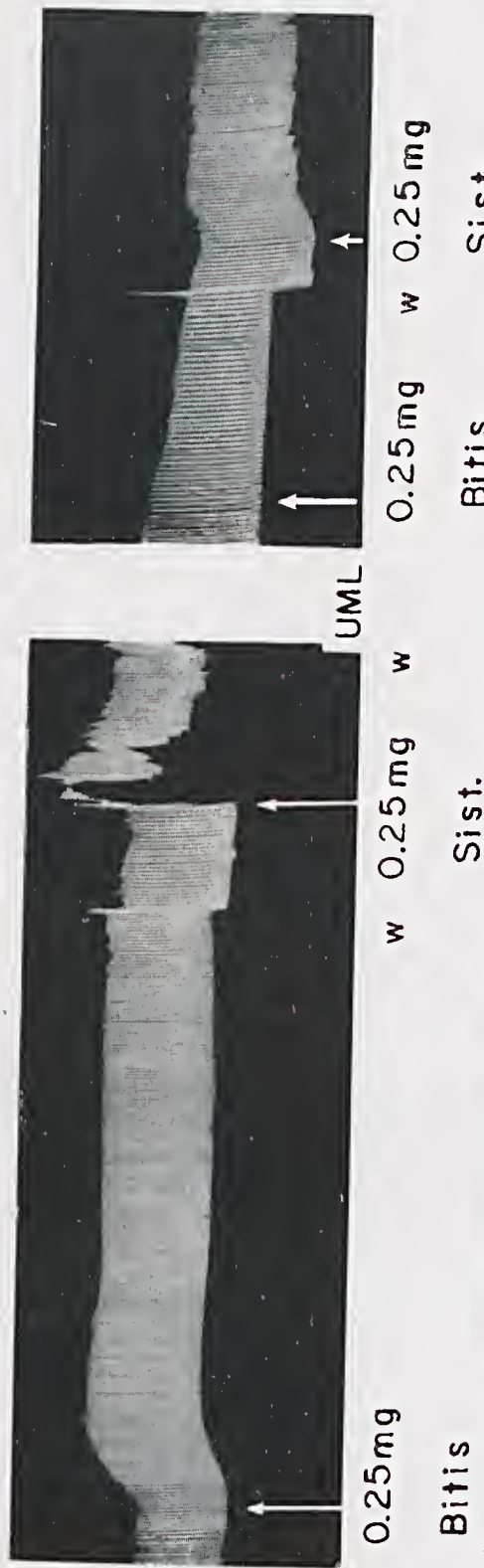
Fig. 1 — Record of bioassay of venoms on the isolated *Mercenaria* heart for presence of serotonin-like activity. Methysergide (UML) added to bath at break in the record. Response to *Bitis gabonica* venom is like that produced by serotonin and completely blocked by UML. Response to *Sistrurus* (Sist.) venom is similar to that produced by a high concentration of serotonin and is only partly blocked by the UML.

Referring to Table II, it may be seen that, of the six elapid venoms tested, an exciter action on the *Mercenaria* heart was found in *Bungarus*, *Hemachatus* and *Pseudechis* venoms but these were not typically serotonin-like. Of the seven viperid and crotalid venoms tested on the *Mercenaria* heart, all but that from *Crotalus horridus* had some serotonin-like exciter action.

In some venoms, notably those of *Pseudechis*, *Sistrurus*, *A. piscivorus* and *Bothrops atrox*, a catecholamine or histamine may be present but we have made no attempt to look for these compounds.

The results from the bioassays are only indicative of the presence of serotonin or related substances and they become significant only when viewed in the light of the results from spectrofluorometric and chromatographic examination of the same venoms.

SPECTROFLUOROMETRY — In most instances, only one sample of venom of a given species was extracted and its fluorescence and excitation spectra recorded with a spectrofluorometer. With the instrument that was used, the uncorrected fluorescence peak for serotonin in 3 *N* HCl appears at 540 mμ and the excitation peak at 305 mμ (Fig. 2). Venoms of 14 species so examined showed no peaks corresponding with those for serotonin. Sample records of recorded spectra of three of these species are reproduced in Fig. 2.

Extracts of venoms of four of the VIPERIDAE showed excitation peaks or shoulders at 305 mμ but a strong fluorescence at 450 mμ obscured the characteristic 540 mμ peak for serotonin (see, for example, records for *Bitis gabonica*, Fig. 2). In addition to the excitation peak or shoulder at 305 mμ, *Bitis* and *Sistrurus* venoms gave a second excitation peak near 280 mμ. This might be due to tryptamine or one of its metabolites that came through the extraction procedure (see results from chromatography).

Only 10-25 mg of each crude venom were extracted for the spectrofluorometric analysis. If some venoms contained low lewels of serotonin the characteristic excitation peak might not have been detected. Certain viperid and crotalid venoms, however, do give evidence for the presence of serotonin by this method.

CHROMATOGRAPHY — The results of only one chromatographic procedure which was used for all of the venoms (except *Bothrops* venoms from Butantan) will be reported. This involved the use of ascending paper chromatography with 10-hours development in butanol acetic acid:H_2O (60:15:25). Serotonin and other indole-reacting spots were made visible by spraying with p-dimethylaminocinnamaldehyde (10). This DMCA reagent is more sensitive but less selective than Ehrlich's reagent. The numbers of indole-reacting spots, made visible by this procedure, are given in Table II. Of the six elapid venoms, three gave one spot each; two gave none; one was doubtful. Chromatograms of seven viperid and crotalid venoms showed from two to five spots made visible by the DMCA reagent.

In an attempt to identify the components of the venoms responsible for these several spots, serotonin and certain related compounds were spotted with the venoms. The following known substances were used: tryptophan, 5-hydroxytryptophan, tryptamine, 5-hydroxytryptamine (serotonin), bufotenine, N-methyltryptamine, N,N'-dimethyltryptamine, indole, indolyl-3-acetic acid, and melatonin.
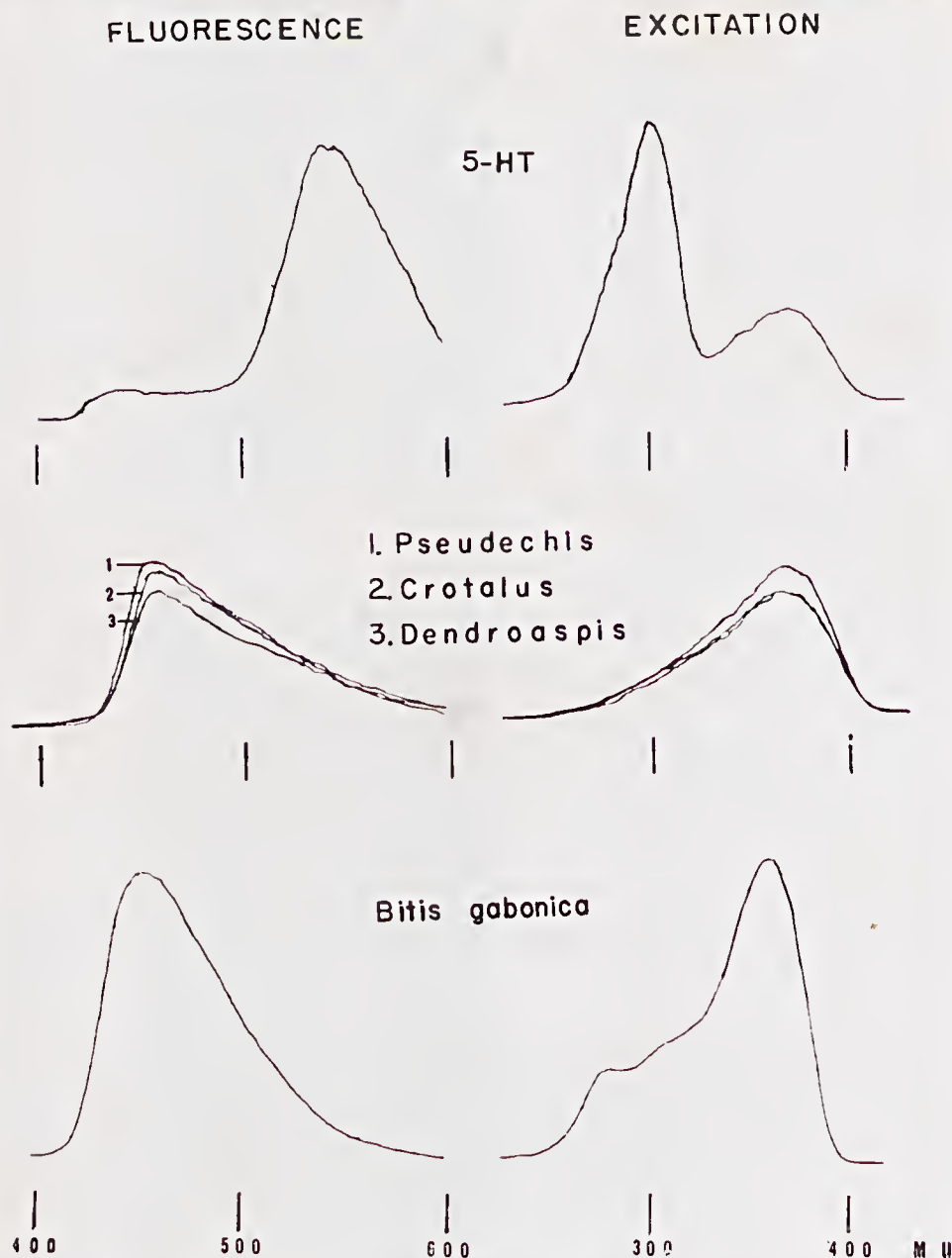
Fig. 2 — Examples of fluorescence and excitation spectra of a serotonin (5-HT) standard of three venoms showing no detectable 5-HT; and of *Bitis gabonica* venom with a characteristic excitation peak at 305 mμ. See text for further detail.

An example of a chromatogram of *Sistrurus* venom run with six of the standards is reproduced in Fig. 3. The venom had been extracted with 50 per cent acetone and volumes equivalent to 10 and 20 mg of venom spotted. The venom may be seen to have yielded two prominent and two faint spots made visible by the DMCA reagent.
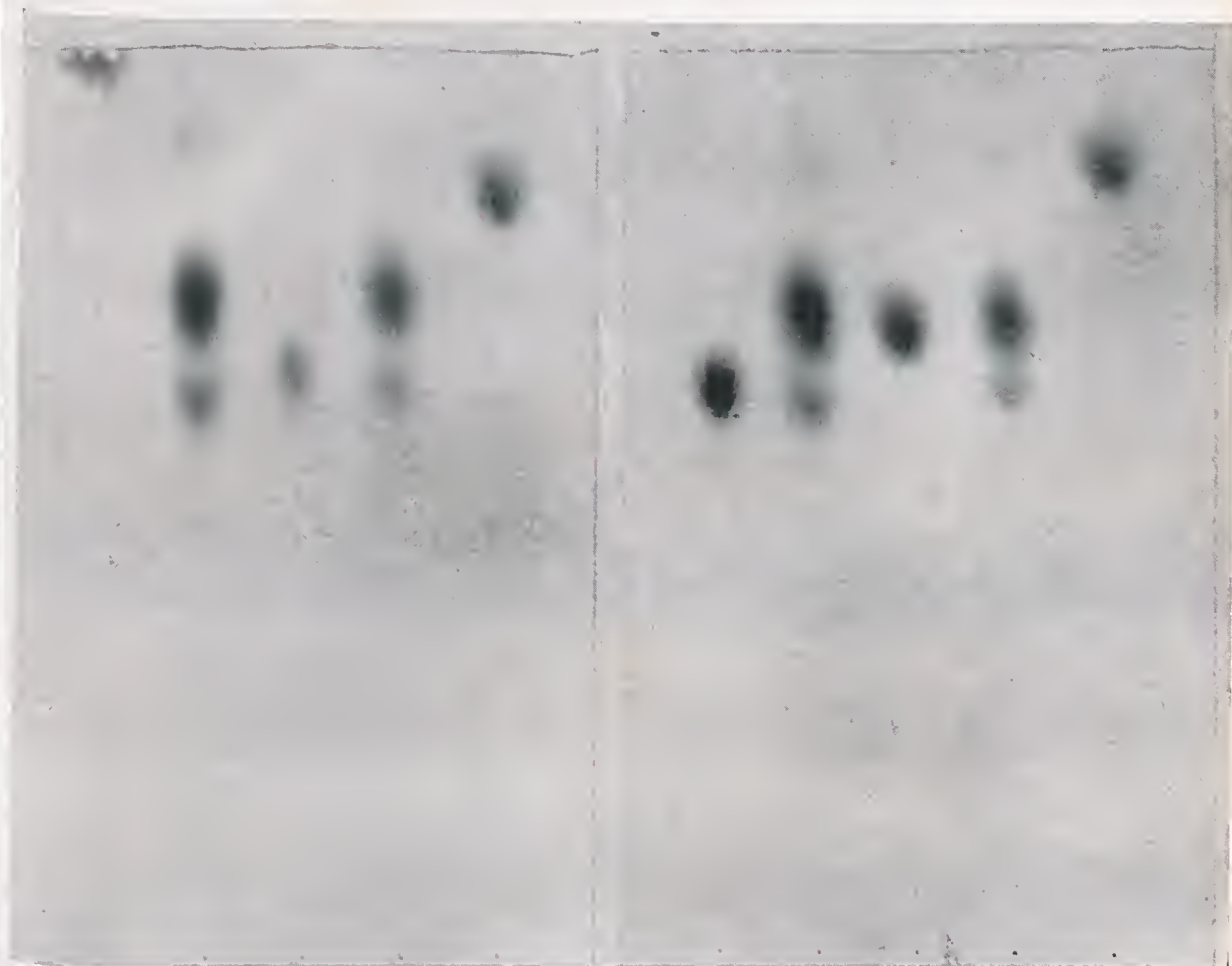
Fig. 3 — Chromatogram of *Sistrurus* venom (10 and 20 mg) run in butanol; acetic acid: water with 5 μg amounts of each of the following standards: melatonin (Melat); tryptophan (T-phan); tryptamine (T-mine); serotonin (5-HT); bufotenine (Bufo) and N-methyltryptamine (N-CH_3-T). Sprayed with DMCA reagent. This chromatogram was developed for 12 hrs and the Rf values are higher than those for a run of optimal duration (8-10 hrs).

The lowest venom spot has an Rf corresponding closely with that for serotonin (5-HT). The next higher, and most prominent, venom spot is at a level near that of bufotenine. There is a faint venom spot at the same Rf as N-methyltryptamine. However, this solvent system fails to separate this amine from N,N'-dimethyltryptamine. A fourth faint venom spot is seen near the front. Melatonin runs to this position but so do indole and indolyl-3-acetic acid.

Chromatograms of the venom of *Agkistrodon piscivorus* (water moccasin) gave four indole-reacting spots that correspond with those from *Sistrurus*

venom and, in addition, a fifth, late-appearing spot with an Rf between 30 and 40. This is close to where 5-hydroxytryptophan runs in butanol:acetic acid:water. With *A. piscivorus* venom (20 mg) the fast-running spot at Rf 95 was much more obvious than with *Sistrurus* venom (20 mg): it gave a purple color with the DMCA reagent which is also true for indolyl-3-acetic acid. *Agkistrodon contortrix* (copperhead) venom (20 mg) gave no spot at Rf 95. The other four spots corresponded closely with those from *A. piscivorus* venom.

An insufficient variety of developing systems have been tried, thus far, to permit the certain identification of any of the indole-reacting substances in snake venoms investigated in this study. It will be necessary to use additional solvent systems and a wider variety of standard indole derivatives before the identity of serotonin and related substances in any given snake venom is more certainly determined.

Discussion

Keele and Armstrong (1), in their book *Substances Producing Pain and Itch*, discuss the constituents of snake venoms that contribute to production of pain. From their citations, pain production at the site of the bite would appear to be more common among the viperids and crotalids than among the elapids. Bradykinin releasing factors constitute one class of agents responsible for pain production in snake venoms. They state (p. 237) "Snake venoms do not contain ACh, 5-HT or histamine, though many of them release histamine from tissues." We now know that venoms of three species of *Dendroaspis* contain large amounts of acetylcholine (3), and from the results of the present study it is apparent that some snake venoms contain a variety of indole-reacting compounds, one of which is probably serotonin. This is a highly potent pain-producer. However, Armstrong has found that tryptamine and certain of its derivatives other than serotonin are also very effective producers of pain when applied to cutaneous nerve endings. Among these are 4-hydroxytryptamine, N-methyltryptamine, N,N'-dimethyl-5-hydroxytryptamine (bufotenine), and N-methyl-5-hydroxytryptamine. The last is even more effective than serotonin (5-hydroxytryptamine). From the chromatographic results of the present study it appears that some snake venoms may contain at least three of these active tryptamine derivatives, namely serotonin, bufotenine, and N-methyltryptamine. If serotonin and bufotenine are present in some venoms, then the intermediate, N-methyl-5-hydroxytryptamine, may also be present. It will be of much interest if further study reveals that some snake venoms do, in fact, contain an assembly of tryptamine derivatives known to be among the most active in producing cutaneous pain in man.

Acknowledgements — Mytolon was supplied by the Sterling-Winthrop Research Institute and methysergide by Sandoz Pharmaceuticals. Carolyn S. Batty, Joyce B. Zipf and Lois D. Williams assisted in this study.

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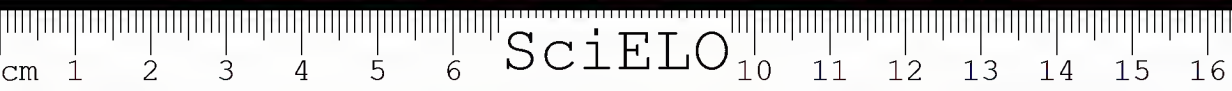
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Discussion

P. Sawaya: "1. When you mention wasps, do you mean the European wasps or the Brazilian wasps from which you have isolated the venoms? 2. The different venoms that you have investigated, how many have a curare-like action?"

J. H. Welsh: "1. The results pertaining to histamine, acetylcholine and kinin referred to the European wasp and hornet venoms. I examined Brazilian wasp venoms only for the presence of 5-hydroxytryptamine. 2. Most of the invertebrate venoms that we have studied have a paralysing action on suitable test animals. However, with the exception of those containing tetramine this paralysing action is probably due to proteins and not to the low molecular weight components."

F. E. Russell: "Would you care to speculate on the mode of action by which serotonin produces pain, perhaps with special consideration of sodium exchange and the possibility of vasoconstriction about sensory endings? Secondly, how might you explain the observation that the serotonin content of two of the snake venoms, you noted *Bitis gabonica* and *Vipera russellii*, while having a 15 plus fold difference in serotonin content have no such marked difference in their pain producing effect in clinical cases?"

J. H. Welsh: "1. The precise mechanism by which serotonin produces pain is not known to me but, at least in some invertebrates, it is known to produce repetitive firing of certain sensory neurons (e.g. the crayfish stretch-receptor sensory

cells). 2. Serotonin is undoubtedly not the only pain-producing factor in snake venoms. Where it is absent, yet the bite is painful, some other factor(s) must account for this."

C. Y. Lee: "Did you find whether there is any cholinesterase activity in *Dendroaspis* venom?"

J. H. Welsh: "By means of bioassays of *Dendroaspis* venoms before and after incubation we found no loss of acetylcholine activity. This suggests that cholinesterase is absent from these venoms which agrees with the results of earlier investigators."

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55. PHARMACOLOGICALLY AND BIOCHEMICALLY ACTIVE COMPONENTS OF JAPANESE OPHIDIAN VENOMS

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We have been making a systematic study on the separation of enzymes of venoms from Japanese and Formosan snakes, and many enzymes were obtained in a pure state.

The most characteristic symptoms produced by injection of the venom of *Agkistrodon halys blomhoffii*, called "Mamushi" in Japanese, a representative of the Japanese poisonous CROTALIDAE snake, are: hemorrhage, necrosis, muscular degeneration and lowering of blood pressure.

Among the enzymes from the venom of *Agkistrodon halys blomhoffii*, one of its three proteinases had hemorrhagic activity, and three were arginine ester hydrolases with "bradykinin releasing", "clotting" and "capillary permeability increasing" activities, they were considered to be connected with the pharmacological actions of the venom. Besides the pharmacologically active enzymes, a striking hemorrhagic protein deprived of any enzymatic activity was also present in the venom. This paper deals with the pharmacologically active components of the venom of *Agkistrodon halys blomhoffii*.

Most of the caseinolytic activities was distributed in three fractions which were designated, in the order of their elution from the column of DEAE-cellulose, as proteinase A, B, and C. Hemorrhagic activity was found mainly in tubes 100 to 170 and 170 to 260. There was little proteolytic activity in the first fraction, designated as HR-I, while in the second fraction, HR-II, there was proteinase B activity. Proteinases A and C were not hemorrhagic factors and no hemorrhage was observed even when they were injected at 300 fold the minimum hemorrhagic dose of proteinase B. When HR-I was injected into a mouse intravenously, marked intestinal hemorrhage was observed, while on injection of HR-II, petechial hemorrhage was observed in the subcutaneous tissues. Next, we attempted to purify HR-II to see whether the hemorrhagic activity was due to the proteinase activity. After HR-II fraction, namely the proteinase B fraction, was desalted by a Sephadex G-25 column, the sample of proteinase B was re-chromatographed on DEAE-cellulose using gradient elution. The curve of absorbancy at 280 mμ did not coincide with the curve of hemorrhagic or proteinase activity, these latters being found in a single peak. After lyophilization, the sample was applied to a hydroxylapatite column, and the hemorrhagic activity was eluted together with the caseinolytic activity. To purify the resulting proteinase B preparation, DEAE-Sephadex A-25 column chromatography was used. By this procedure some impurities were removed. The hemorrhagic and caseinolytic activities of proteinase B were not separated by these purification procedures and

the increase in potency of the homorrhagic activity at each step was essentially in parallel with that of the caseinolytic activity of proteinase B. The average yields of the purified proteinase B, from crude venom was about 2.5 per cent. The purified preparation was chromatographically, electrophoretically and also ultracentrifugally homogeneous.

Proteinase B showed to be a metal-protein, and the hemorrhagic and caseinolytic activities fully remained even after the removal of sialic acid by sialidase. But its activities were inhibited by EDTA and cysteine. In EDTA-inhibition experiments, the extent of the decrease in hemorrhagic activity was parallel with the decrease in caseinolytic activity. Also, in cysteine-inhibition experiments, the hemorrhagic activity decreased parallel with the caseinolytic activity, in proportion to the amount of cysteine added.

There was another hemorrhagic fraction, namely the fraction of HR-I containing arginine esterases, from which the arginine esterases were easily removed by hydroxylapatite chromatography. The HR-I preparation thus obtained contain no enzyme activity. Although the protein content of this HR-I preparation was low, the toxic principle must be a protein and when this was treated with various proteinases, the hemorrhagic activity decreased.

As the hemorrhagic activity of HR-I appeared to play a leading role in the lethality of the venom of *Agkistrodon halys blomhoffii*, a comparison of the hemorrhagic and proteinase activities in various venoms were examined. The crude venom of *A. contortrix contortrix* had no hemorrhagic activity even though it had high proteinase activity. On the other hand, the venom of *Crotalus adamanteus*, which showed strong hemorrhagic activity had low proteinase activity. Moreover, when the latter venom was fractioned on a DEAE-cellulose column, 98 per cent of the total hemorrhagic activity was recovered in a fraction which had no proteinase activity. This hemorrhagic factor was pharmacologically different from HR-I isolated from *Agkistrodon halys blomhoffii* venom. So, it seems that even in venoms of the same family, there may be different hemorrhagic factors and different substances with lethal toxicity, and for the characterization of these toxic principles, it is essential to purify each factor from each snake venom.

In the same way, we purified two kinds of arginine esterases, namely the "clotting" and the "capillary permeability increasing enzymes" in physico-chemically pure states. We also obtained the bradykinin releasing enzyme, free from other physiologically active components.

As a considerable amount of the "clotting enzyme" and the "capillary permeability increasing enzyme" were present in the venom, we attempted to purify the first to a physico-chemically homogeneous state. Purification procedures consisted of four steps and by these procedures the clotting enzyme was purified to a physico-chemically homogeneous state.

Among the arginine ester hydrolases in the eluate from the DEAE-cellulose column, an enzyme which has a hypotensive action and increases capillary permeability was found. The arginine ester hydrolytic activity of this enzyme was 30 to 40 per cent of the total arginine ester hydrolytic activity of the venom, and when it was injected into the skin of an albino rabbit, the permeability of the capillaries were distinctly increased as shown by the Evans Blue Test.

When this preparation was incubated with purified bradykininogen, no release of bradykinin was detected by assay on guinea-pig ileum. So, it is not clear by what mechanism the permeability of the capillaries is increased by this enzyme.

In other experiments, we found that a considerable amount of this capillary permeability increasing enzyme was also present in *Crotalus adamanteus* venom and in *Trimeresurus flavoviridis* venom. This enzyme of the venom of *Agkistrodon halys blomhoffii* was purified by similar procedures as those which were used for the clotting enzyme. The specific activity of this enzyme was more than 70-fold that of the crude venom, and the purified preparation was homogeneous on ultracentrifugation and cyanogum electrophoresis at various pH values.

The ultracentrifugation patterns of the "clotting" and "capillary permeability increasing" enzymes show the homogeneities of the enzymes.

When the purified "clotting enzyme" was incubated with 98 per cent pure fibrinogen, which had been prepared in our laboratory, according to the method of Blombäck and Laki, three kinds of fibrinopeptides were liberated. Two of these corresponded to fibrinopeptides A and B which were liberated from fibrinogen by the action of thrombin or by the venom of *Bothrops jararaca*. But. one of the three peptides was a new fibrinopeptide. By amino acid analysis and end group analysis of this new fibrinopeptide, it was concluded to be a peptide which resulted from the loss of one mole of arginine from the C-terminus of fibrinopeptide B. The reason why the physico-chemically pure "clotting enzyme" of the venom, which showed only arginine ester hydrolytic activity, liberated this new peptide is not yet known. When the enzyme was incubated with fibrinopeptide B, no reaction was observed.Therefore, the new fibrinopeptide seemed to be released directly from the bovine fibrinogen molecule by the action of the clotting enzyme.

The clotting activity of the enzyme of *Agkistrodon halys blomhoffii* was lower than that of the clotting enzyme of *Bothrops jararaca*. The activity of the clotting enzyme of *Agkistrodon halys blomhoffii* was not inhibited by plasma anti-thrombin. In this it is similar to the clotting enzyme of th evenom of *Bothrops jararaca.*

Bradykinin releasing enzyme fraction which was obtained from the eluate on the first DEAE-cellulose column chromatography of *Agkistrodon halys blomhoffii* venom, contained clotting enzyme. And it was further applied to a CM-cellulose column. Thus the bradykinin releasing enzyme, free from clotting enzyme, was obtained, but only 5 per cent of the total units of arginine ester hydrolytic activity of the venom were recovered in this partially purified enzyme preparation. Therefore, no further purification was attempted. Although the substrate specificities of the clotting, bradykinin releasing, and capillary permeability increasing enzymes were qualitatively the same, their physiological activities were completely different. The bradykinin releasing was only inhibited by trasylol, which is a potent inhibitor of urinary and pancreatic kalliereins, and the clotting and capillary permeability increasing enzymes were not inhibited by trasylol at all. From these results and the specificities found by tests on several synthetic substrates, the bradykinin releasing enzyme in the venom seems to be a salivary kallikrein of the snake.

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56. SPECIFIC SITES OF ACTION OF SNAKE VENOMS IN THE CENTRAL NERVOUS SYSTEM

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Introduction

Neurotoxins from snake venoms are their active components responsible for disturbances in the central and peripheral activities of the nervous system. These disturbances are produced by the venom of all species of the family ELAPIDAE, such as the Indian Cobra (*Naja naja*), all Australian venomous snakes (1) and *Walterinnesia aegyptia*, the only ELAPIDAE native to Israel (2). Neurotoxins are also components of the venom of the American pitvipers, CROTALIDAE (3) and have been isolated from the venoms of many VIPERIDAE, although hemorrhagic and cytolytic activities predominate in the action of the latter venoms (4).

In our previous publications we have described the electrophoretic (5) and chromatographic (6) separation of neurotoxic fractions from *Vipera palestinae* venom. The administration of these fractions to mice produces neurotoxic signs and causes death of the animals without hemorrhages taking place. The hemorrhagic fraction produces widespread bleeding in mice, but is devoid of any neurotoxic action.

Distinct electrophoretically separated neurotoxic fractions from the venom of the Indian Cobra (*Naja naja*) have also been reported (7), but the relations between the toxic and enzymatic activities in this and other venoms have not been finally elucidated. Phospholipase A, protease, L-amino acid oxidase, cholinesterase, nucleases, phosphodiesterase, monoesterase, hyaluronidase have been found in most venoms and some of them, nucleases (8), cholinesterase (9), phospholipase A (10) have been suspected to cause various envenomation syndromes. Above all, the phospholipase A of the Indian cobra (*Naja naja*) venom has been considered to possess neurotoxic activity (10) although in recent publications this assertion is being dissented (7, 11).

The present series of experiments was undertaken as an attempt to elucidate the specific action of these neurotoxins on brain mechanisms, autonomic functions and their toxicity to different species.

Materials

Whole venom from adult *Vipera palestinae* specimens was obtained from the Institute of Natural Science of the Tel Aviv University (Prof. M. Mendelssohn and Dr. E. Kochwa, to whom thanks are due). Neurotoxic and hemorrhagic

fractions were separated as previously described (12, 6). Maximum sublethal dosis, equivalent to 40 mouse LD_{50} units of venom or venom fraction, per kg body weight, were administered intravenously to cats and rabbits.

Indian Cobra (*Naja naja*) freeze dried venom from L. Light and Co. Ltd., Colnbrook. England was used.

Phospholipase A from *Naja naja* venom was obtained by paper electrophoretic separation at pH 6.0, using phosphate buffer M 15. After electrophoresis a narrow segment (of about 1 cm width) was cut off from the middle of each strip (about 7 cm width) and stained with naphthalene-black. Fig 1 shows 6 bands
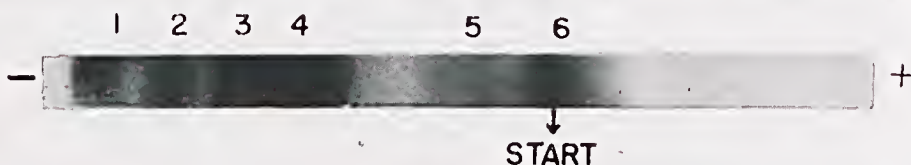
Fig. 1 — Paper electropherogram of *Naja naja* venom. Conditions as described in "METHODS".

in the electropherogram. The appropriate protein bands were cut off from the unstained strips and eluted over night with distilled water at 4°. Sodium chloride was added until isotonicity and protein concentration was estimated by Lowry reaction (13). Only the fraction remaining at the application line (fraction 6) contained the phospholipase A activity. The adjacent fraction 5 proved in most separations to be devoid of phospholipase A activity but in a few instances was contaminated with the enzyme. The phospholipase A containing fraction 6 was tested for the following activities: protease (1), procoagulant (13), L-amino acid oxidase (14) and hyaluronidase (15) and was found devoid of all of them. Analytical ultracentrifugation of fraction 6, dialyzed against saline, was carried out with a model E, Spinco ultracentrifuge at 16.5°, using the standard 12-mm cell, and a single peak was obtained corresponding to a sedimentation constant $S_{20} = 3.65 \times 10^{-13}$. Fractions 1 and 2 contained the direct lytic factor, a basic protein, capable to lyse washed red blood cells (12).

For some experiments the venom was boiled and treated with heparin. For this purpose a saline-venom solution, 1 mg/1 ml, was heated for 15 min in a boiling waterbath at pH 5.5 and then centrifuged. The electrophoretic pattern of the supernatant was similar to that of the unboiled venom i.e. comprising 6 bands. 1 mg heparin (National Biochemicals Corporations, Heparin-sodium 100 U/mg) in 0.1 ml saline was added to 1 ml of the supernatant and the mixture was kept for 20 min at room temperature. A sediment appeared which is known to contain the direct lytic factor (12). It was separated by centrifugation and electrophoresis of the clear supernatant showed that fractions 1 and 2 were absent.

Phospholipase A activity

One ml of tenfold saline diluted egg yolk was incubated at 37° for increasing periods of time with 0.05 ml venom or phospholipase A fraction containing 0.5 μg protein. The unesterified fatty acids (UFA) were determined by titration using the method of Dole (16). The activity was calculated as m-equiv. of free

acid released per enzyme fraction per minute. For experiments only those phospholipase A preparations were used which liberated at least 1.6 m-equiv. UFA/min per 1 μg protein.

Methods

Experiments on mice

Intraperitoneal mouse-LD_{50} of whole venom and its separate fractions were determined on locally bred Swiss albino mice, using 5 animals per dose. Calculation of LD_{50} was made according to Reed and Muench (17).

Experiments on cats

The experiments were carried out on anaesthetized intact cats and spinal cats, weighing 2-3.5 kg. Anaesthesia was induced by ether and maintained throughout the experiment by repeated injection into a femoral vein of 0.5-1 ml of a mixture containing 10 mg thiopentone sodium and 40 μg atropine sulfate per ml. Blood clotting was prevented by intravenous administration of heparin (5,000 units/ml/kg body weight).

All activities studied were recorded on a Grass model 5 polygraph. Blood pressure was registered from the cannulated femoral artery using a Statham transducer. Respiration was measured by means of a thermocouple introduced into the cannulated trachea. The electrocardiogram was obtained using needle electrodes inserted into the extremities. Peripheral circulation was studied by photoplethysmography, using a Hoffman 55-c silicone photocell with high sensitivity in the infrared range, placed around the cat's paw. Variations in intensity of the light reaching the sensitive area of the photocell on transillumination of the exposed extremity are a measure of variations in the amount of blood in the light path and thus indicate vasoconstriction (decreasing volume) or vasodilation (increasing volume) (18). The direct current obtained from the photocell was amplified by a Grass D.C. amplifier. In order to facilitate the recording of prolonged changes in blood volume, amplification was reduced to a point where pulse waves almost disappeared.

Electrocorticograms (EcoG) were recorded bipolarly using insulated stainless steel electrodes with bare tips placed in contact with the brain surface through holes drilled in the skull on both sides in the frontal, temporal and occipital areas.

Changes in autonomic nervous system activity were detected by recording the action potentials of a few fibres of the left cervical sympathetic chain. The fibres were dissected from the main nerve trunk and mounted on platinum wire electrodes which were isolated except for the immediate area in contact with the nerve fibres. The preparation was immersed in physiological saline solution at 37° covered with liquid paraffin. The electrodes were connected to a Tektronix type 132 low level preamplifier and the nerve potentials continuously viewed on a Tektronix 515 A oscilloscope. A record of the potentials was also stored on a Grundig TK 45 tape recorder for delayed transcription.

Spinal cats were prepared according to the method of Dale (19). Artificial respiration was started using a Starling pump and the brain was destroyed with a probe introduced through an orifice made in the second cervical vertebra;

bleeding was controlled by inserting a tapering pack of gauze into the brain space. Ether anaesthesia was maintained until the destruction of the cerebral tissue was accomplished.

The contraction of the nictitating membrane produced by preganglionic stimulation of the ipsilateral cervical sympathetic chain was recorded simultaneously with blood pressure in cats before and after administration of the neurotoxin. Hexamethonium was used as a control. Depression of the response of the nictitating membrane occurring simultaneously with a fall in blood pressure was regarded as indicative of ganglion blocking activity in the tested compounds.

Experiments on rabbits

Conscious rabbits, under local anaesthesia (novocain) only, and weighing 2-3 kg, were fitted with a stereotaxic apparatus according to the method of Monnier and Gangloff (20). Recording electrodes were inserted into holes drilled over the left and right frontal and parietal lobes of the cerebral cortex, and stimulating electrodes insulated except for their tips were placed in the midbrain reticular formation.

Electrocorticograms were recorded on a Grass electroencephalograph, and stimuli in the reticular formation were produced with a Grass stimulator model S4 with an attached isolation unit, and monitored with a Tektronix model 502 oscilloscope.

The cortical arousal response was induced by stimulation of the reticular formation; the voltage of the stimulus varied between 0.2-1.5 volts, the duration was 0.5 msec, and frequency was 240 pps. Variation in the threshold and duration of the evoked cortical response, as well as changes in the electrocorticogram (ECoG) itself after injection of the venoms were noted.

Forty rabbits were used in this study, each experimental group consisted of a minimum of 5 animals. The venoms and their fractions were injected intravenously at a dose of 40-50 mouse LD_{50} per kg, causing death within 1-2 hours after injection.

Experiments on the isolated frog Sciaticus nerve

Studies on the influence of snake venoms and their separated neurotoxic fractions on the action potential of isolated nerve preparations were carried out *in vitro*. A length of 7-8 cm of frog sciaticus nerve was cut out and dissected from the spinal cord and the main trunks were further separated from the smaller branches. By threads connected to both ends, the nerves were supported in a nerve chamber, consisting of three separate compartments with a small guiding groove for the nerve passing through all the compartments. The nerve was placed upon two platinum electrodes in each of the three compartments. Transmission was observed by stimulating one end of the nerve in the first compartment and recording the provoked action potential at the other end in the third compartment. Changes in threshold during the experiment were found by stimulating the middle part of the nerve which was in direct contact with the toxins.

A Grass 4 stimulator and a Tektronix 502 oscilloscope were used for stimulation and recording. In some cases the action potentials were photographed using a polaroid camera.

Results

A — *Vipera venom*

1. *Experiments on cats*

Four cats were injected with whole *Vipera palestinae* venom and sixteen with the neurotoxic fraction. In order to obtain a survival period of at least four hours after the first injection, maximal sublethal doses were used. The following phenomena were observed both after whole venom and neurotoxic fraction administration. A sharp fall in blood pressure took place within 20 s. after injection. This reduction in blood pressure continued throughout the duration of the experiment in most of the animals, although in some cases the blood pressure remained constant at the initially established lower level. The pulse pressure diminished progressively to a very low amplitude. The fall in blood pressure was associated with continuously progressive peripheral vasodilatation, as shown in Fig. 2, although a decrease in the peripheral blood volume may be observed for a short period while the acute blood pressure drop is taking place, due to acutely impaired blood supply.
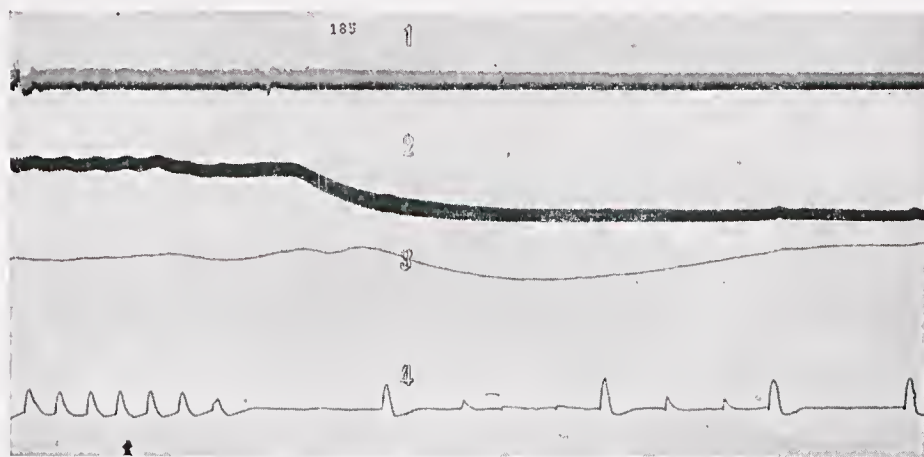
Fig. 2 — Pharmacodynamic effect of *Vipera palestinae* neurotoxin. Order of tracings from top to bottom: ECG, blood pressure, photoplethysmography, respiration. For explanation, see text.

Respiration showed decreased frequency and increased volume, with brief periods of apnea (Fig. 2); later, the respiratory pattern is superficial and slow. The ECG record showed little change; the voltage decreasing continuously during the experiment.

The electrical activity of the central and autonomic nervous system was strongly affected. ECoG voltage was diminished in all leads and sometimes entirely abolished for short periods, three minutes after the injection of venom or neurotoxin (Fig. 3). In all cases the action potentials of the cervical sympathetic

chain disappeared completely within 30 s. after injection (Fig. 3 *B*). It appears, therefore, that sympathetic firing was erased much earlier than ECoG waves; also the latter were suppressed incompletely or only for a limited period of time (Fig. 3 *C*).
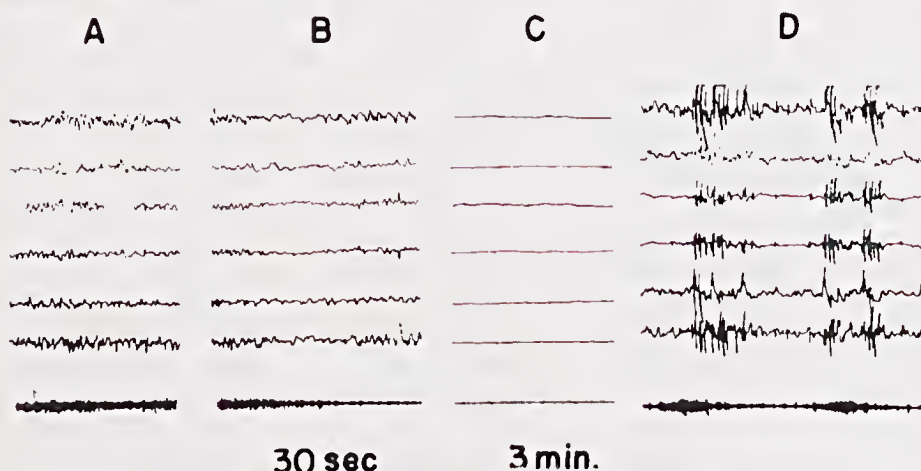
Fig. 3 — Effect of *Vipera* neurotoxin on the ECoG and spontaneous activity of the cervical sympathetic chain. Order of tracings from top to bottom: ECoG (frontal, parietal and occipital leads) and sympathetic nerve action potentials. A: before, B: 30" and C: 3' after neurotoxin injection. D: 80 min. after injection. The nerve action potentials disappear much earlier than the ECoG.

Six cats recovered from the first injection of *Vipera palestinae* neurotoxin or whole venom, showing decreased but steady patterns of blood pressure, respiration and electrocorticogram. A second injection of the same dose of neurotoxin or of whole venom was given to these animals up to 30 min following the first dose. This second administration of venom did not provoke a further decrease in blood pressure. Tachyphylaxis was not observed when the first injection consisted of the neurotoxic fraction and the second was whole venom. In this case, blood pressure again dropped sharply and immediately.

Doses of 20 mouse LD_{50} units of the neurotoxin, equivalent to half the maximum sublethal dose for intact cats, were administered intravenously to spinal cats. During 2-3 min. after the injection a slow increase in blood pressure amounting to 10 to 20 mm Hg was observed. The increased level was maintained for about ten minutes, after which a return to the previous level took place. ECG, recorded simultaneously with the blood pressure, was not affected and the cardiac rhythm was unchanged. In six cats, the administration of 20 mouse LD_{50} units of the neurotoxin did not affect the response of the nictitating membrane to pre-ganglionic sympathetic stimulation, even at the height of the acute blood pressure decrease described previously. In control experiments, hexamethonium injected prior to the neurotoxin in doses of 1 mg/kg, produced a transient lowering of the blood pressure with a simultaneous reversible depression of nictitating membrane contraction.

Injection of maximum sublethal doses of the hemorrhagic fraction of *Vipera palestinae* venom into nine cats caused no alteration in blood pressure in seven of the animals. A slight transient fall in blood pressure was noticed immediately

after injection in the two remaining animals only. The sympathetic nerve potentials were unaffected or only slightly decreased and no changes were observed in the ECoG and peripheral blood volume. Mice injected intraperitoneally with 2 LD_{100} units of the same fraction died within 2 hours, showing wide-spread hemorrhages in accordance with previous observations (12).

2. *Experiments on rabbits*

The injection of 40-50 mouse LD_{50} per kilogram of whole *Vipera* venom evoked a gradual decrease in the amplitude and frequency of the cortical potentials, in several cases finally resulting in complete disappearance of detectable cortical activity. In general no effect was noted on the threshold or duration of the arousal response elicited by stimulation of the reticular formation, although occasionally its duration was prolonged.

3. *Isolated nerve*

Vipera palestinae neurotoxin was also found to depress the provoked action potential in the isolated frog sciatic nerve preparation. Transmission through the intoxicated nerve was completely blocked after incubation with the neurotoxin for 1-1½ hours at a concentration of not less than 150 mouse LD_{50} units per ml. This block was only partially reversible on washing with frog Ringer (see Fig. 6). A response was obtained on direct stimulation of the toxin-incubated portion of the nerve, despite the transmission block. However, in some preparations the excitability threshold was that of the initial threshold.

B — *Cobra venom*

1. *Experiments on mice*

Action of whole Cobra venom

The intraperitoneal LD_{50} for mice was 0.18 mg/kg. Mice injected with $2 \times LD_{50}$ showed convulsions, excitement and impaired movements, and died in respiratory arrest within 80 minutes.

Cobra venom fractions

Of the 6 paper electrophoretic Cobra venom fractions, three-combined fractions 1 and 2, fraction 3 and fraction 6, had neurotoxic activity, although having different species specificity and mechanism of action.

The LD_{50} for the combined eluates of fractions 1 and 2 was about 1.5 mg (protein) per kg body weight. Mice injected intraperitoneally with 2 LD_{50} of this combined eluate showed neurotoxic symptoms — apathy, motor depression, and close to death 3 hours after venom injection, gasping respiration, jumping and clonic convulsions.

Fraction 3 possessed stronger neurotoxic activity, the intraperitoneal LD_{50} being 0.5 mg/kg. Mice injected with this fraction showed excitement, jumping,

convulsions, fast respiration with use of auxiliary respiration muscles, and curling of the tail. Death occurred early, not later than 30 min after the injection.

Animals receiving fraction 4 exhibited in some cases similar symptoms but the LD_{50} was much higher, above 2.5 mg/kg. Fraction 5 was not toxic at the doses injected, up to 2.5 mg/kg. Fraction 6, the only one which contained phospholipase A activity, was not toxic, even at doses of 5 mg/kg. This conforms to the findings of Master and Rao (7) with Indian cobra phospholipase A purified by starch gel electrophoresis.

2. *Experiments on cats*

Cobra venom

The intravenous LD_{100} in anesthetized cats was 1 mg/kg, the animals dying within 2 hours from the moment of injection. In experiments on 12 cats, the injection of one LD_{100} caused a diphasic circulatory shock. Immediately after the injection a transient drop in blood pressure occurred, associated with apnea and bradycardia, high P waves and ST depression (Fig. 4 *B*). Later, after the mean blood pressure had returned to the preinjection level, there occurred a temporary depression in the ECoG. In the period from about 10 min after injection till shortly before death, respiration was fast and pulse pressure decreased progressively, ECoG and ECG remaining normal (Fig. 4 *C*). Near to death, 80-120 min. after the injection (Fig. 4 *D*), the ECoG slowly waned, the respiration became progressively bradypneic until respiratory arrest occurred. Thereafter the blood pressure dropped to zero and the animal died.
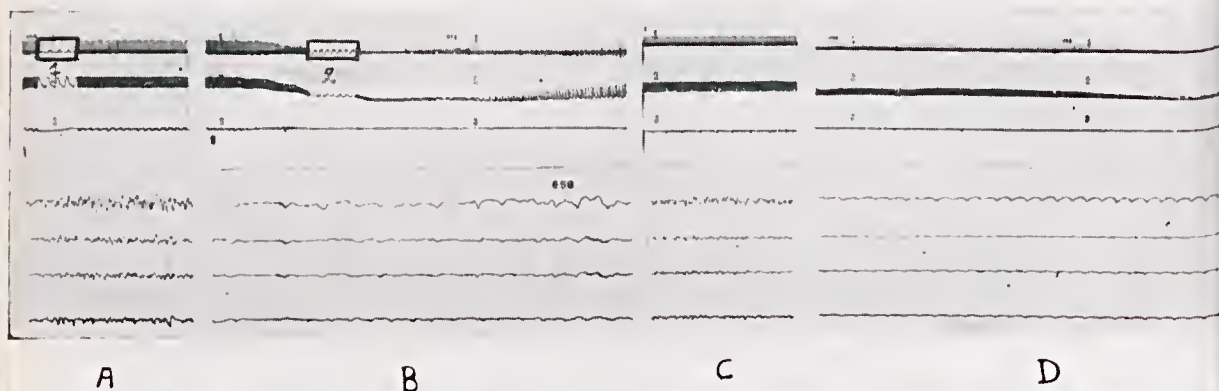
Fig. 4 — Effect of Cobra venom (1 LD_{100}) in an anesthetized cat. Order of tracings (from top to bottom) ECG, blood pressure, respiration and ECoG — frontal, parietal (two leads), occipital areas. Record A: normal tracing before venom injection; B: immediately after venom injection; C: 30 min after injection; D: 80 min after injection at time of death.

Recording of the action potentials from the cervical sympathetic chain showed increased firing starting shortly after venom injection, gradually increasing in frequency and amplitude, up to the time of death. Increased sympathetic firing together with maximally depressed ECoG 10 min. before death were noticed. At this time bradypneic respiration persisted, the sympathetic firing increasing at inspiration.

Cobra venom fractions

Combined fractions 1 and 2 were injected into 2 cats intravenously in the amount of 1 mg (protein)/kg. One of the cats showed transient changes in the ECG-lowering of QRS voltage and ST elevation, lasting for about 10 min. after injection. No other toxic effects were recorded.

Fraction 3 was given to 3 cats intravenously at doses of 0.25 mg/kg (1 LD_{100}). No changes were seen in blood pressure, ECG, ECoG and respiration at the moment of venom injection (Fig. 4 *A*), but 120 min. later ECoG slowly waned (Fig. 4 *B*), respiration became sporadic and then stopped completely. Pulse pressure diminished progressively during the two hours following injection and fell to zero. During the period of apnea the ECG showed increasing bradycardia and anoxic changes (Fig. 4 *B*).

Fraction 4 was as a rule non-toxic, but sometimes symptoms similar to those produced by fraction 3 could be elicited at higher doses (0.8 mg/kg), probably due to deficient separation from fraction 3.

Fraction 5 preparations were found non-toxic, even in high doses up to 1.5 mg/kg, when devoid of phospholipase A. In those cases in which fraction 5 possessed phospholipase A activity by contamination from fraction 6, the preparation did show neurotoxic activity.

Fraction 6, which had strong phospholipase A activity, was neurotoxic, the LD_{100} being approximately 1 mg/kg. The pharmacological effect was identical to that of the whole Cobra venom with the two typical shock phases described above.

The activity of phospholipase containing boiled-heparinized Cobra venom was indistinguishable from that of fraction 6 and of whole venom. The LD_{100} was similar, about 1 mg/kg.

3. *Experiments on rabbits*

Cobra venom

The injection of whole venom caused an early quickened respiration which was later replaced by respiratory difficulty and gasping. Death occurred due to respiratory arrest which was preceded by clonicotonic convulsions. Earlier, partial paralysis of the limbs, particularly the hind legs, was noted.

Immediately after injection, there was a much increased cortical activity and awareness, as shown by a strong beta rhythm in the electrocorticogram. This was accompanied by quickened respiration and hypermotility. Within the following 10-15 minutes, respiration considerably slowed, limb paralysis began to appear, and the cortex was characterized by a gradually increasing depression of activity, as evidenced by a steadily increasing delta rhythm in the ECoG. (Fig. 5-3a). This state of cortical depression and labored respiration was maintained for up to 2 hours. About 10 minutes before death, the animals underwent great respiratory stress, characterized by slow gasping respiration. At this time, strong convulsions began to appear, but these convulsions were not accompanied by characteristic seizure patterns in the electroencephalogram. Cortical potentials then waned and completely disappeared (Fig. 5, 5a); only after complete disappearance of the ECoG record did death occur due to respiratory arrest.
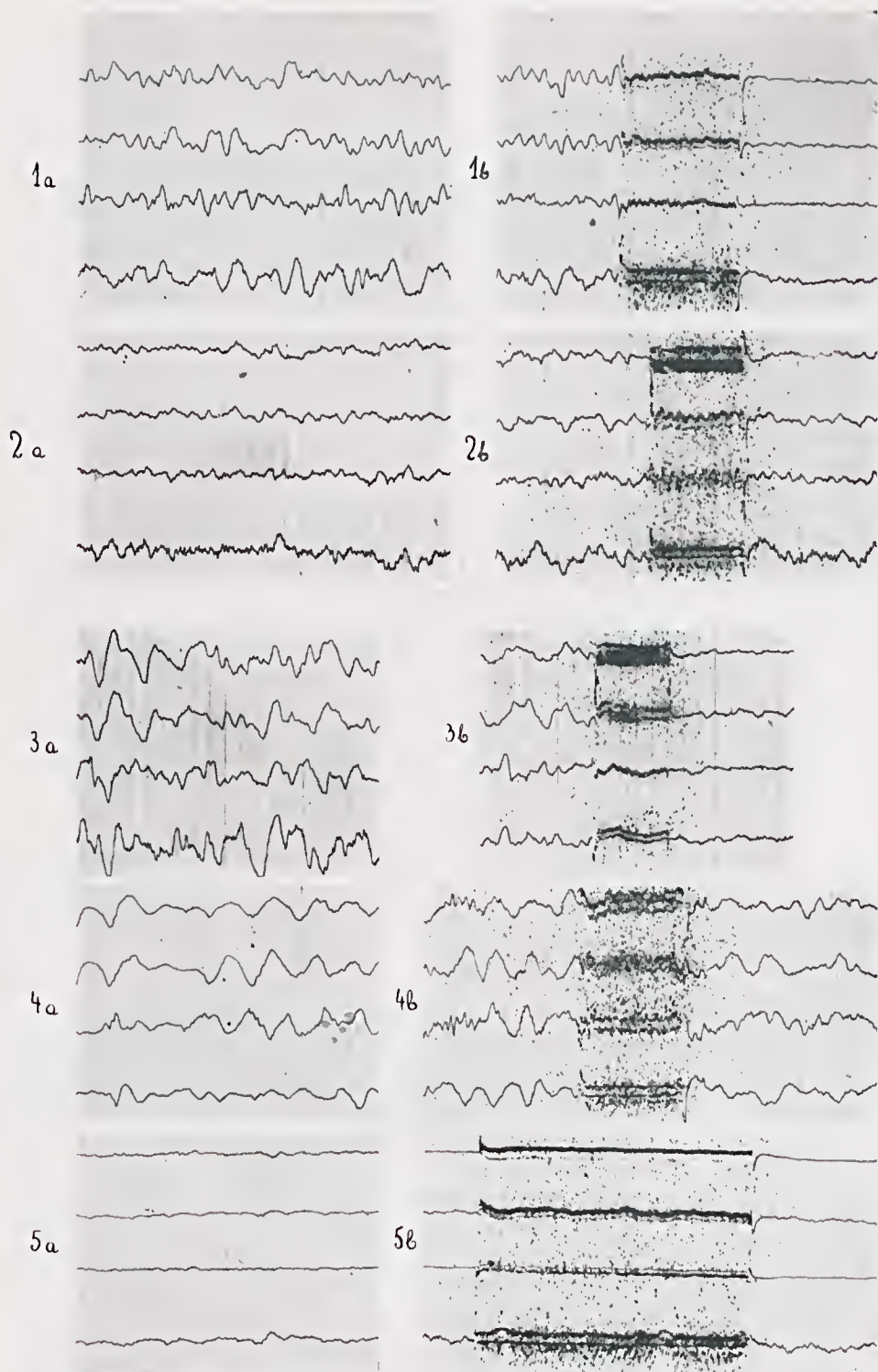
Fig. 5 — ECoG Record. Left side — ECoG before and after venom injection. Right side — Cortical arousal response elicited by direct stimulation of the reticular formation. 1 a, b — before injection; 2 a, b — immediately after injection; 3 a, b — 20 minutes after injection; 4 a, b — 60 minutes after injection; 5 a, b — 70 minutes after injection.

The arousal response threshold also changed characteristically upon the injection of the venom. During the initial period of excitation following the injection, the threshold was elevated. It then gradually decreased to a point below that determined before the injection, and remained at this low level for the majority of the remaining time, paralleling temporally the appearance of cortical depression. Shortly before death (Fig. 5, 4b) and before the total flattening of the ECoG waves, the arousal response could no longer be elicited even with relatively high voltage stimulation.

Cobra venom fractions

Three of the electrophoretically-separated protein fractions showed neurotoxic activity, namely fractions 1-2, 3 and 6.

The effects caused by fraction 1-2 were similar to those of whole venom, except that the cortical depression was much less marked and that the terminal flattening of the ECoG potentials was pronounced and occurred much earlier in this fraction than in others. The arousal response disappeared only after a complete ECoG flattening; until the point of its disappearance, the threshold remained lower than the control level and coincident with cortical depression.

Fraction 3 was the most toxic of the fractions. Its neurotoxic action differed from that of whole venom in that the cortical depression was very marked and pronounced, and persisted until almost immediately before respiratory arrest when the cortical potentials finally disappeared. The arousal response disappeared much earlier than with any other fraction, in the midst of the period of cortical depression (delta rhythm).

Fraction 4 and 5 possessed no neurotoxic activity. No change was observed in the ECoG or the arousal response after injections, and the animals in this group remained alive at least four hours after injection.

Fraction 6, on the other hand, caused almost identical effects as the whole venom.

4. *Isolated nerve*

All three neurotoxic fractions blocked conduction through the isolated frog Sciaticus nerve, in a pattern and concentrations similar to those described for the *Vipera* neurotoxin (see Fig. 6).

Non neurotoxic fraction 4-5 were inactive, even at a concentration of 2 mg/cc.

Discussion

The electrical activity of the cervical sympathetic chain reflects the state of the medullary cardiovascular centres which regulate blood pressure by controlling peripheral arteriolar sphincters. Consequently, sympathetic firing is decreased when these centres become depressed (21). During blood pressure fall due to causes other than depression of the medullary centres, sympathetic firing is increased (22, 23), probably due to compensatory feedback mechanisms.
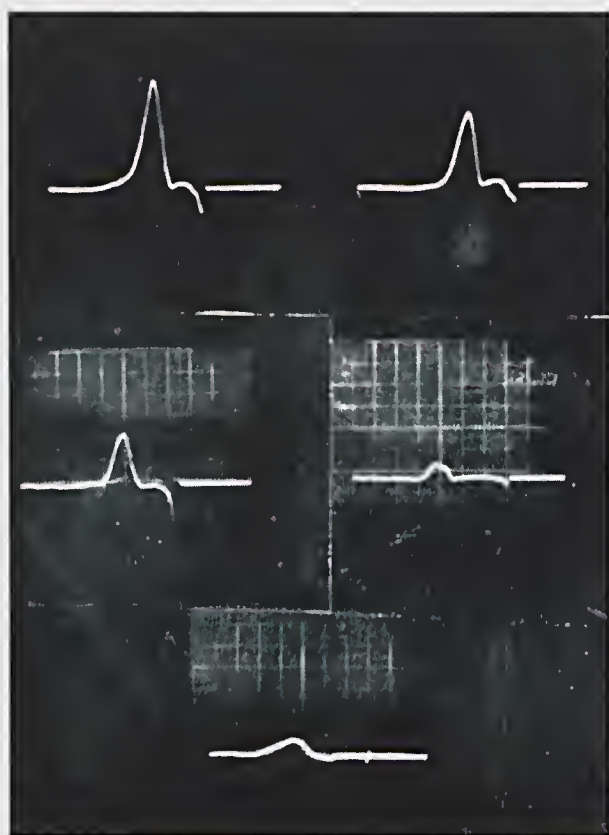
Fig. 6 — Effect of neurotoxins on the evoked frog sciatic nerve action potential. The electric response (upper left) is progressively depressed (upper right after 30', middle left after 60', middle right after 80'), and disappears completely after 90' incubation. Bottom tracing: after 2 h washing in frog Ringer, partial recuperation is seen.

Vipera palestinae whole venom as well as its separated neurotoxic fractions produce immediate suppression of the cervical sympathetic action potentials, affecting the electrical activity of the cortex only later. In addition, the marked and prolonged rise of the photoplethysmographic record indicates increased blood volume in the dilated peripheral vascular bed.

The present results have also shown that *Vipera palestinae* neurotoxin has no cardiotoxic or ganglion-blocking activity. It seems probable that the slight transient blood pressure rise observed in the spinal cat is produced by a moderate vasoconstriction induced by the toxin.

The instantaneous depression of blood pressure associated with simultaneous inhibition of sympathetic action potentials and peripheral vasodilatation indicate that a primary action on medullary vasopressor centres may be responsible for the neurovegetative effects of *Vipera palestinae* whole venom or neurotoxin.

ELAPIDAE (*Naja naja*) and CROTALIDAE (*Crotalus atrox*) venoms are also known to cause an immediate and severe fall in systemic blood pressure. According to earlier observations (24, 25), cobra venom, when administered in high doses, produce two phases of circulatory shock, an initial fall in blood pressure, attributed to histamine release in lung tissue with concomitant obstruction of pulmonary circulation, and late respiratory failure. Lower doses of *Naja naja* venom produce neurotoxic effects without appreciably affecting cardiovascular activity. Experiments carried out in our laboratory showed that the initial blood pressure decrease as a result of intravenous administration of cobra venom to cats and associated with immediate disappearance of ECoG waves is not associated with depression of cervical sympathetic action on potentials (26).

The precipitous fall in arterial blood pressure caused by the venom of *Crotalus atrox* was shown by Russell *et al.* (27, 3) not to be due to depression within the central nervous system, but to a deficit in left heart output secondary to changes in resistance in the pulmonary circulation.

Cardiovascular effects and shock have also been described in relation to venoms of other animals. *Latrodectus* venom (28) was found to have a direct action on the heart and coronary circulation. Stonefish venom impairs the contractile force of the heart and produces vasodilatation not of central origin (29).

It therefore seems warranted to assume that medullary cardiovascular centres are specific receptor sites for *Vipera palestinae* neurotoxin but not for the venoms of some other poisonous snakes, spiders or fish. Consequently, the mechanism of the primary cardiovascular shock produced by each of these venoms is different. The blocking of peripheral nerve transmission and lowering of its excitability indicate a direct toxic action of *Vipera palestinae* neurotoxin on the nerve membrane.

The hemorrhagic component of *Vipera palestinae* venom does not produce primary shock and has no influence on the sympathetic nerve potentials. This fraction produces bleeding in experimental animals, which eventually leads to death.

Earlier studies by Houssay (30) suggested a correlation between the neurotoxic activity of CROTALIDAE venoms and their hemolytic action. Feldberg and Kellaway (24) attributed the steep fall in blood pressure after intravenous injection of Cobra venom to lecithinase which, by producing lysolecithin, liberates histamine from the tissues. Bragança and Quastel (10) reaffirmed the identity of neurotoxin and cobra venom phospholipase A. Cobra venom heated for 15 min. at 100° retained both its neurotoxic and phospholipase A activities while other enzymes were inactivated. On the other hand, phospholipase A-containing fractions, separated from various snake venoms by electrophoresis or chromatography, were recently reported to be devoid of neurotoxic activity, the latter being recovered in other fractions (6).

In the present study an animal specific action of different paper electrophoretically separated *Naja naja* venom neurotoxins, only one of them — fraction 6 — having phospholipase A activity, was demonstrated. This was apparent not only from the observed resistance of mice to fraction 6 and of cats to fractions 1-2, but also from the different syndromes appearing in cats and mice. Fraction 6 produced in cats diphasic circulatory shock, depression of respiration, eventually leading to respiratory death, and early occurring ECoG depression and changes

in sympathetic activity. Fraction 3 provoked early death and convulsions in mice, whereas in cats delayed respiratory arrest and death with late occurrence of similar electrophysiological response.

The diphasic circulatory shock ensuing in cats following intravenous administration of whole *Naja naja* venom, as well as of fraction 6 and of heated heparinized venom (phospholipase containing, devoid of direct lytic factor (12) and of the mice-neurotoxic activities related to fractions 1-2 and 3) resembles that described by Feldberg and Kellaway (24) for whole *Naja naja* venom. After recovery from the immediate steep drop in blood pressure, there was a late occurrence of a secondary gradual descent until respiratory death supervened. Fraction 3 did not produce diphasic shock but only a late lethal effect appearing synchronously with the second part of the diphasic shock caused by whole venom or fraction 6. Thus, whereas in whole venom-induced intoxication the phospholipase A-containing fraction 6 plays a role in the causation of both shock phases, fraction 3, which is devoid of phospholipase A, contributes to the delayed shock phase only.

Although the primary shock has been attributed by Feldberg and Kellaway to the histamine liberating action of the venom, a direct action of the venom phospholipase A on the central nervous system, manifested in our experiments by transient ECoG depression and changes of respiratory rhythm, cannot be excluded.

It was established in the present study that *Naja naja* venom provokes increased cervical sympathetic action potentials with primary depression of ECoG waves. In contradistinction, *Vipera palestinae* venom neurotoxin has been shown to reduce cervical sympathetic firing immediately upon injection without simultaneously affecting the cortical activity (31). It appears therefore that neurotoxins from different snakes act on different sites in the central nervous system.

Phospholipase A from different snake venoms have been shown to have different substrate specificity. The phospholipase A of both cobra and *Vipera palestinae* venoms hydrolyse phospholipids in soluble state, such as in egg yolk and plasma. However, whereas the cobra phospholipase A readily attacks the phospholipids in human osmotic red blood cell ghosts (12) and blood platelets (32), cat brain homogenates and cat brain mitochondria (33), the *Vipera palestinae* phospholipase A has no such activity. This may reflect a possible biochemical correlation with the difference in neurotoxicity of the separated phospholipase-containing fractions of the *Naja naja* and *Vipera palestinae* venoms, the former being toxic to cats, the latter non-toxic to both mice and cats (6).

A specific action of cobra venom phospholipase A on nervous membranes was demonstrated by Tobias (34), who used the isolated lobster giant axon. The inactivation of the action potentials was not accompanied by electron microscopically demonstrable membrane changes. The assumption of a direct central action of *Naja naja* phospholipase A, however, as yet lacks an *in vitro* corrolary, since the enzyme, which is able to split phospholipids in brain homogenates, does not act on intact brain slices (33).

Although several investigators have demonstrated a peripheral curare like action of cobra venom neurotoxin, a central neurotoxic activity has also been studied. Westerman *et al.* (35) demonstrated abolition of a central vagal reflex and Guyot (36) observed neurotoxic symptoms when the venom was injected intraventricularly, and Ciuchta (37) and others have demonstrated changes in the ECoG of intoxicated animals. Whether these effects are primary results of neurotoxic activity or are secondary to other physiological changes is still unclear.

Since it was demonstrated that the animals continued to respire even after cortical potentials could no longer be detected, it may be assumed that the central action of cobra venom neurotoxin is a primary action of the venom itself; that the observed neurotoxic effects are not secondary to respiratory difficulty and caused simply by anoxia to the brain tissue. This is not to say that the respiratory arrest is necessarily caused by the same central-acting components, but it seems clear that a strong contributory cause, if not the main underlying factor, in the death due to cobra venom intoxication is the deleterious effect of the toxin on the brain and the disappearance of cortical activity.

It was noted above that fraction 1-2 caused early disappearance of cortical potentials as evidenced by complete flattening of the ECoG record. Furthermore the arousal response threshold was not significantly elevated during the period of cortical depression. and disappeared only after the disappearance of cortical activity. These two facts tend to indicate that this neurotoxic fraction has a particular affinity to the cortex itself, and lower centers are affected only later and more weakly.

According to the same line of reasoning, it was concluded that fraction 3 acts particularly on the reticular formation and perhaps other lower centers. As the arousal response disappeared long before flattening of the ECoG became apparent.

Fraction 6, whose action paralleled that of whole venom, seems to act on both cortical and subcortical structures, although the action is first on the cortex and later on the reticular formation. This seems to elaborate our previous observation that this phospholipase A-containing fraction is toxic for cats, although it is non-toxic in mice as demonstrated by Master and Rao (1961) (7).

The fact that the terminal convulsions observed were not accompanied by the characteristic spike, and some patterns usually noted in seizures as well as the observation that often the convulsions appeared in the midst of the period of ECoG delta rhythm (paralleling cortical depression) seem to indicate that the convulsions observed are of subcortical origin.

The negative findings that the venom of *Vipera palestinae* has no effect on the arousal response seems to lend support to a specificity in central action of this neurotoxin, selectively depressing central autonomic vasoregulatory mechanism.

It may be concluded from the above work that these neurotoxins tend to act on specific centers in the central nervous system, and that neurotoxic action is generally not random and diffuse. Nevertheless, it is clear that all nervous tissue is to some extent vulnerable to neurotoxic action, although specific affinities too clearly seem to exist.

The locus of action of *Vipera* neurotoxin seems to be more restrictive and definitive than that of *Naja naja* neurotoxin. This perhaps is due to its predominant action on more primitive centers, as opposed to the predilection of cobra venom for the higher centers.

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Discussion

C. Y. Lee: "Is there any evidence that cobra neurotoxin, which is a basic polypeptide, can pass through the blood-brain barrier in sufficient quantity to produce central effects?"

H. I. Bicher: "There are some publications using radioactively labelled venom, that indicate that small amounts of cobra venom reach indeed the central nervous system. The specificity of the observed electrophysiological effects makes us suppose that this small amount may be enough for a delimitated effect. Similar experiments by Gotter and his co-workers demonstrated that *Vipera palestinae* venom also can penetrate the blood brain barrier."

J. C. Vidal: "With the fraction (neurotoxin of 6th band) have you found hydrolysis products due to phospholipase A activity?"

H. I. Bicher: "We did not test ourselves, but people of our Institute, and specially Dr. Klivansky and Dr. Condrea demonstrated phospholipid splitting *in vivo*. However, we think that the action of the neurotoxic fraction is a direct one upon the CNS cells, and not mediated through the liberation of chemical products in the blood stream. May be so at the cell membrane level."

57. ACTION NEUROMUSCULAIRE DES VENINS DE QUELQUES *CROTALIDAE*, *ELAPIDAE* ET *HYDROPHIIDAE*

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Dans les trois grands groupes des serpents venimeux, OPISTOGLYPHES, PROTEROGLYPHES, SOLENOGLYPHES, les deux derniers sont pratiquement les seuls dangereux pour l'Homme.

Parmi les signes de l'intoxication on note pour beaucoup une action neuromusculaire. Nous nous sommes attachés à l'étude de cet effet produit par les venins de:

PROTEROGLYPHES

ELAPIDAE	*Naja*	*Naja naja*
		Naja haje
		Naja nigricollis
HYDROPHIIDAE	*Lapemis*	*Lapemis hardwickii*
	Hydrophis	*Hydrophis cyanocinctus*
	Enhydrine	*Enhydrine schistosa*

SOLENOGLYPHES

CROTALIDAE	*Crotalus*	*Crotalus durissus terrificus*
		C. d. terrificus
		C. d. terrificus var. *crotaminicus* (crotaminé)

Pour respecter l'ordre chronologique de nos recherches, nous examinerons d'abord les effets des venins de Crotale et la crotamine, puis les venins des *Naja* et des HYDROPHIIDAE, l'action inhibitrice neuromusculaire de ces deux derniers groupes étant voisine.

Les quantités réduites de venins mises à notre disposition nous ont obligé à chercher des préparations les économisant le plus possible.

Les schémas de ces essais comprennent:

la toxicité,

les essais sur le muscle strié, stimulation indirecte par le nerf, excitation directe du muscle,

préparation tibial antérieur de Rat *in situ*, innervé ou dénervé,

préparation nerf sciatique, muscle tibial antérieur, muscle soléaire *in situ* de Chat,

préparation diaphragme isolé de Rat innervé ou dénervé,

préparation du *rectus abdominis* isolé de Grenouille,

parfois une courte incursion sur le muscle lisse,

préparation d'iléon isolé de Cobaye.

VENIN DE *CROTALUS DURISSUS TERRIFICUS* var. *CROTAMINICUS* ET CROTAMINE

(Venins provenant de l'Institut Butantan (Dr. Schenberg), crotamine fournie par le Pr. J. Moura Gonçalves).

Ils ont des actions qualitativement semblables, ils seront donc traités ensemble.

TOXICITÉ (250 mcg/kg crotamine inject. i/v queue de la sourie blanche), la crise a un aspect typique d'une durée de 2 à 3 minutes, on constate une contracture du dos avec rejet des pattes postérieures en arrière, les doigts des pattes antérieures recroquevillés sur eux-mêmes. L'animal se déplace en rampant sur les moignons de ses pattes antérieures ainsi que sur sa queue. Les crises se reproduisent plusieurs fois. Entre temps il y a une accélération respiratoire et une démarche anormale "en canard" sur la pointe des doigts des pattes postérieures.

PRÉPARATION NEUROMUSCULAIRE IN SITU DE RAT

ACTION PROPRE DIFFÉRENTE SELON LES DOSES:

25-50 mcg/kg i/v, pas de modification de l'amplitude des contractions mais diminution de l'aptitude à mantenir un tétanos.

100-200 mcg/kg, le muscle répond aux excitations mais il y a gêne à la décontraction jusqu'au niveau de base. On note une tachyphylaxie par répétition des doses, fait dejà signalé par les auteurs brésiliens.

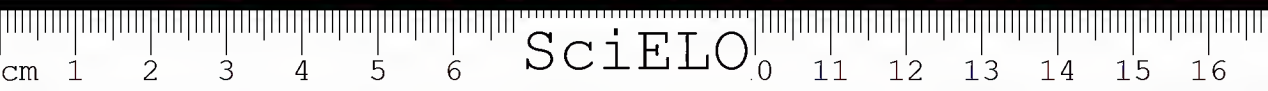
400-500 mcg/kg, donne phénomène complexe:

a — une forte contracture avec forte élévation du niveau de base, gêne de la décontraction et diminution de l'amplitude des contractions tant par stimulation indirecte (nerf) que par stimulation directe (muscle).

b — retour à la ligne de base initiale et diminution de l'amplitude.

c — retour à l'amplitude du départ et même augmentation de celles-ci par rapport à la normale.

d — durée phénomène: 30 minutes environ, puis après temps variable (30-45 min.) apparaissent des contractures spontanées moins fortes mais typiques.

e — une nouvelle injection est sans effet.

Le fait que les résultats sont identiques sur les mouvements entraînés par excitation indirecte ou directe *est en faveur d'une origine musculaire du phénomène.*

Préparation dénervée (tibial antérieur dénervée). Sur un tel muscle excité directement une injection de crotamine produit une contracture mais elle est moins forte que celle entraînée par stimulation du nerf de l'autre patte. De plus la tachyphylaxie apparaît dès la 2.ᵉ injection sur le muscle dénervé. *Ceci est aussi en faveur d'une action directe sur le muscle.*

Interaction avec les paralysants neuromusculaires — Nous avons utilisé un acétylcholinocompétitif (d-tubocurarine), un acétylcholinomimétique (C_{10}).

d-tubocurarine — La contracture par la crotamine se produit comme si la préparation n'était pas curarisée, mais dans un 2.ᵉ temps les 2 substances agissent pour donner une inhibition complète avant retour des mouvements (Fig. 1).

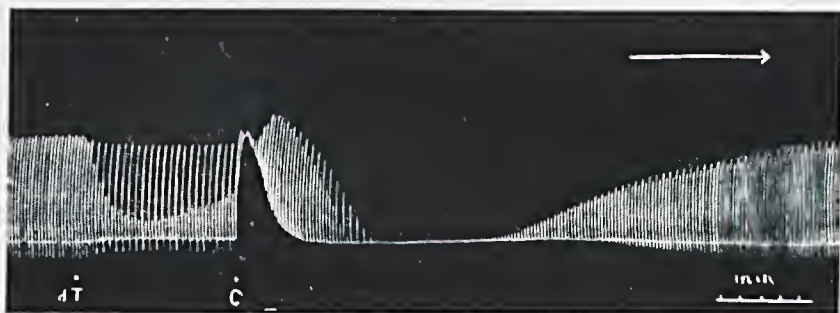



Fig. 1 — RAT: Contractions du muscle tibial antérieur *in situ*. Stimulation alternée du nerf (indirecte) et du muscle (directe) toutes les 10 secondes (nerf sciatique: 0,2 msec, 3 V — muscle 0,1 msec, 60 V). En dT: *d*-tubocurarine — 40 mcg/kg i/v: seule la contraction indirecte est déprimée. En C: Crotamine — 500 mcg/kg i/v: contracture typique avec reprise fugace des contractions indirectes puis inhibition complete des deux contractions.

Le maintien de la contracture après curarisation est un argument de plus pour une action musculaire.

La crotamine diminue la sensibilité à une 2.ᵉ injection ultérieure de d-tubocurarine.

décaméthonium — Après C_{10}, la contracture est maintenue mais est moins forte que sur une préparation non partiellement inhibée. La crotamine augmente la sensibilité à une 2.ᵉ injection de C_{10}.

Antagonisme des ions —

Antagonisme des ions Ca^{++} — Comme pour la vératrine l'injection de $CaCl_2$ annule la contracture crotaminique et la prévient si $CaCl_2$ est injecté préalablement.

Antagonisme des ions Mg^{++} — L'action est du même type qu'avec Ca^{++}, ils sont moins toxiques mais leur effet est plus fugace.

TACHYPHYLAXIE CROISÉE — Nous avons montré une tachyphylaxie croisée entre crotamine et venin à crotamine et vice-versa. Par contre le venin sans crotamine et la crotamine administrés l'un après l'autre et vice-versa n'ont entraîné aucune atténuation de la contracture crotaminique. *La tachyphylaxie serait donc due à cette dernière substance.*

PRÉPARATION NERF PHRÉNIQUE — DIAPHRAGME ISOLÉE DE RAT

ACTION PROPRE — Crotamine (7 mcg/ml) entraîne une action contracturante avec élévation du niveau de base et décontraction difficile, la contracture est généralement immédiate ou peu différée. Elle décroit sans lavage, progressivement mais reparaît par lavage. Puis des contractures apparaissent spontanément à intervalles longs imprévisibles.

ACTION DES IONS — Ca^{++} prévient la contracture ($CaCl_2$ 200 mcg/ml) celle-ci apparaît au lavage. Ca^{++} supprime la contracture en cours. Mg^{++}-d°-mais abolit plus difficilement une contracture en cours.

INTERACTION AVEC LES PARALYSANTS NEUROMUSCULAIRES

d-tubocurarine (dose entraînant une faible paralysie). La crotamine entraîne une faible contracture qui apparaît nettement au lavage.

décaméthonium, après très faible contracture par crotamine et lavage n'entraîne pas de contracture importante.

TACHYPHYLAXIE CROISÉE obtenue sur ce test par venin à crotamine et crotamine ou vice-versa.

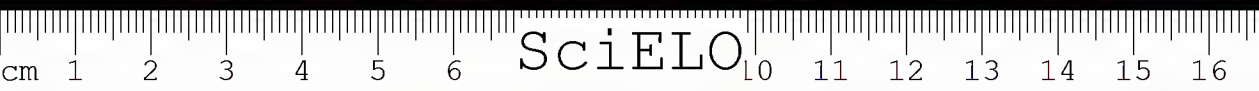
RECTUS ABDOMINIS DE GRENOUILLE

ACTION PROPRE — Aucun effet contracturant sur ce test.

2 à 5 mcg/ml de crotamine sensibilise considérablement le *rectus* au K^{+} doublant ou triplant les effets du K^{+} (30 à 45 min. après), cette sensibilisation persiste après lavage et se prolonge 12 heures et plus. Les ions Ca^{++} (250 mcg/ml) suppriment les effets de la crotamine vis à vis du K^{+}.

10 mcg/ml de crotamine doublent les effets de l'acétylcholine, mais cette action disparaît après lavage.

ILÉON DE COBAYE

La crotamine (2,5 mcg/ml) provoque la contracture des fibres lisses avec apparition des mouvements spontanés de la fibre lisse, disparaissant par lavages répétés. Peu ou pas d'action sensibilisante à l'acétylcholine.

VENIN DE *CROTALUS DURISSUS TERRIFICUS* SANS CROTAMINE

TOXICITÉ — 250 mcg/kg i/v chez la Souris provoquent une gêne respiratoire de toutes les souris après 5 minutes. 50% meurent en 24 heures. Ce venin est donc plus toxique que celui à crotamine, conclusion donnée également par Vital-Brazil qui pense que la toxicité du venin du *C. d. terrificus* est due à la crotoxine.

PRÉPARATION NEUROMUSCULAIRE *IN SITU* DE RAT — Après une légère augmentation de l'amplitude des contractions au moment de l'injection, on observe une paralysie de la préparation très lente à s'établir et *irréversible*, les myogrammes sont identiques sur le tibial antérieur excité directement comme sur le tibial excité indirectement par son nerf. *Il semble donc que le venin agisse directement sur le muscle.*

Très léger et fugace antagonisme par Ca^{++} et chlorhydrat de Choline. Après venin sans crotamine le venin à crotamine exerce son effet contracturant habituel si la paralysie n'est pas trop avancée, mais l'effet est atténué. Le venin sans crotamine sensibilise aux inhibiteurs neuromusculaires tant d-tubocurarine que succinyldicholine.

Si sur une préparation neuromusculaire de Chat, on injecte dans l'artère tibiale de l'acétylcholine, on a une contraction plus grande ou égale à celle donnée par l'excitation indirecte par le nerf, après le venin l'effet acétylcholinique est diminué. *Donc les récepteurs de la plaque motrice paraissent également touchés.* Sur le diaphragme isolé de Rat on note une paralysie progressive lente et irréversible.

RECTUS ABDOMINIS DE GRENOUILLE — Après un certain temps (doses $\geq$ à 10 mcg/ml) on note des contractions de grande amplitude mais de courte durée atténuables ou supprimables par les ions Ca^{++}. Pas des modifications des sensibilités au K^{+} ou à l'A/C.

L'action contracturante de la crotamine est-elle vératrinique? Ce rapprochement a été présenté par Moussatché et Gonçalves en 1956. Quatre points sont communs entre les effets de ces deux substances:

1 — Mêmes symptômes d'intoxication chez la Souris.

2 — Sensibilisent le *rectus abdominis* de Grenouille aux K^{+} (c'est l'effet veratrinique de BACQ).

3 — Gênent la décontraction du muscle.

4 — Ont leurs effets antagonisés par les ions Ca^{++} et Mg^{++}.

Par contre d'autres effets sont nettement différents. Entre autres:

1 — Sur une préparation neuromusculaire non stimulé, la crotamine provoque une contracture, la vératrine ne le fait pas.

2 — Sur une préparation neuromusculaire stimulée directement ou indirectement, isolée ou *in situ*, la vératrine provoque une augmentation de l'amplitude des contractions sans élévation de la ligne de base alors que la crotamine provoque une élévation de la ligne de base et diminution pendant la contracture de l'amplitude des contractions.

3 — La crotamine présente une paralysie secondaire jamais observée avec la vératrine.

4 — La crotamine présente le phénomène de tachyphylaxie et non la vératrine.

5 — Les effets de la vératrine varient en fonctions de la fréquence de stimulation, alors que ceux de la crotamine sont identiques, quelle que soit la fréquence de stimulation.

6 — Après d-tubocurarine (dose entrainant une paralysie partielle), la crotamine produit une contracture puis la paralysie souvent totale. La vératrine entraîne la reprise des contractions et l'augmentation d'amplitude au-delà du niveau d'origine.

VENINS DE *NAJA* (*N. naja, N. haje, N. nigricollis*)

(Venins provenant de l'Institut Pasteur (annexe de Garches — Dr. Boquet, Paris)

Les actions *qualitatives* sont du même type pour les trois, nous décrirons plus particulièrement celles du *N. naja* indiquant à la fin les différences surtout d'ordre quantitatif entre les trois.

TOXICITÉ — L'aspect qualitatif est toujours semblable mais la valeur quantitative assez variable d'un échantillon à l'autre bien que ceux-ci soient conservés à l'état sec en tubes bien bouchés, parfois scellés et en glacière. Chiffres variables. Souris blanche (DL_{50} i/v allant de 390 mcg/kg à 850 mcg/kg). Les solutions sont toujours préparées extemporanément, l'activité diminuant en 24 à 48 heures de façon sensible.

La mort présente toujours le même aspect. Après un temps de latence plus ou moins long, gêne respiratoire qui s'accentue jusqu'à l'asphyxie, le cœur continuant à battre après l'arrêt respiratoire.

Même tableau, chez le Poussin de 8 jours, bien que celui-ci soit plus sensible que la Souris, la DL_{50} i/v se situant vers 100 mcg/kg.

PRÉPARATION NEUROMUSCULAIRE *IN SITU* DE RAT

ACTION PROPRE — Après injection par voie i/v ou i/artérielle, la paralysie s'installe après un temps de latence plus ou moins long, selon la dose administrée, elle est lent (plusieurs heures), progressive et irréversible dans les 7 à 8 heures des expériences.

Si l'on enregistre l'excitation directe et indirecte du muscle, on constate que le muscle reste contractile jusqu'à la fin, même quand l'excitation indirecte par le nerf est inefficace.

Cet aspect est comparable à une curarisation classique, la transmission nerveuse est annulée alors que la contraction directe par excitation du muscle persiste.

ESSAI D'ANTAGONISTES — La néostigmine, l'édrophonium sur une paralysie par venin de *Naja* encore à moitié des contractions normales, assure une reprise nette mais fugace.

Sur une même préparation en cours de paralysie par venin de *N a j a*, le venin de *Crotalus durissus terrificus crotaminicus*, donne sa contracture habituelle, puis une reprise des mouvements, mais la paralysie *progressive* et *définitive* réapparaît après.

INTERACTION AVEC INHIBITEURS NEUROMUSCULAIRES, D-TUBOCURARINE — Alors que l'on peut injecter par voie i/v jusqu'à 6 fois 20 mcg/kg de d-tuboeurarine sans curarisation, les mêmes injections après une seule injection de 100 meg/kg de venin de *N. naja* sont toujours progressivement efficaces.

Avee la succinylcholine les résultats sont contradictoires. *Chose peu étonnante le Rat ne réagissant pas de façon pure aux acétylcholinomimétiques, la première injection agit selon ce type, mais par répétition des doses l'évolution se fait vers le type compétitif.*

CHUTE DE LA TÊTE DU LAPIN

Sur des lapins étalonnés par rapport à la d-tubocurarine ou à la succinyldicholine, nous avons montré qu'une injeetion de venin de *N. naja* (80 meg/kg) diminuait de 50% les doses de d-tuboeurarine nécessaires pour obtenir la chute de la tête et ceci, sensible 9 heures après l'injection du venin se maintient en s'atténuant jusqu'au 9.e jour.

Pour les raisons indiquées ci-avant, les résultats avec la succinyldicholine sont contradictoires.

DIAPHRAGME ISOLÉ DE RAT — Le venin de *N. naja* entraîne après un certain temps de latence une contracture (élevation du niveau de base) disparaissant par lavage. En même temps s'installe la paralysie progressive, lente, irréversible. Néostigmine, édrophonium antagonisent nettement mais passagèrement la paralysie. Les ions Ca^{++} et Mg^{++} agissent de même. Le venin de *C. d. terrificus crotaminicus* donne sa contraeture habituelle, mais la paralysie réapparait inexorable.

Comme pour la préparation *in situ*, le venin de *N. naja* sensibilise le diaphragme à la d-tubocurarine. Avec la succinyldicholine pas de sensibilisation mais parfois antagonisme. Ceci augmente la parenté de l'aetion du venin de *N. naja* avee les acétylcholinoeompétitifs.

DIAPHRAGME DÉNERVÉ ISOLÉ DE RAT — Sur une telle préparation les mouvements provoqués par excitation directe du muscle étant enregistrés si on arrête les mouvements et ajoute de l'A/C durant l'arrêt, on a une eontraction d'amplitude normale. Après addition de venin on voit l'aetion de l'A/C disparaître (Fig. 2). On retrouve ces effets sur une préparation *in situ* de Chat par injeetion d'A/C dans l'artère tibiale avant et après venin de *N. naja*.

On peut donc admettre que le venin de *N a j a* diminue la sensibilité des plaques motrices à l'acétylcholine.

RECTUS ABDOMINIS DE GRENOUILLE — A doses faibles le venin de *N. naja* n'a aucun effet. A doses fortes on obtient des eontractures, ne disparaissant pas par lavage. A la dose de 0,5 mcg/kg, on a suppression des effets de l'aeétylcholine, mais aucune action sur les effets du K^+.

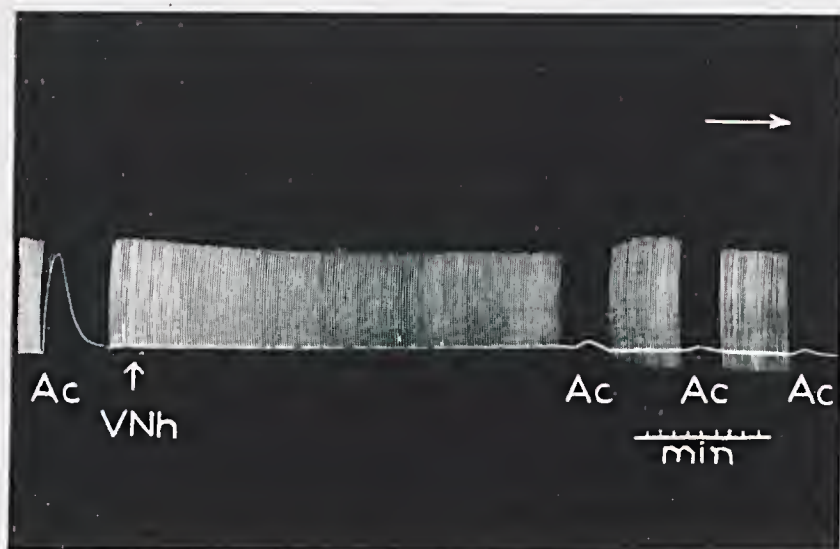



Fig. 2 — RAT: Hémidiaphragme chroniquement dénervé et isolé: Stimulation du muscle (0,1 c/s — 0,1 à 0,2 msec — 60-80 V). La stimulation électrique est interrompue pour permettre l'addition au bain de l'acétylcholine (30 sec) et le lavage continu de la préparation (9,5 min). La stimulation électrique est reprise 5 min avant une nouvelle addition d'acétylcholine. En Ac, l'acétylcholine (0,75 mcg/ml) est ajoutée avant et 30-45 et 60 min après l'addition (en VNh) de 1 mcg/ml de venin de *Naja haje*. Solution de Tyrode — Bain de 50 ml à 36-37°C — Oxygénation par 95% O_2 + 5% CO_2.

Quelques différences entre les trois venins de Naja: Réserve faite des différences d'activité d'un échantillon à un autre, il nous a paru que le venin de *N. haje* était quantitativement le plus paralysant (dose minima active 100 mcg/kg i/v). L'étude neuromusculaire du venin de *N. nigricollis* est difficile à montrer sur l'animal entier à cause de son action cardiovasculaire brutale choquant beaucoup les sujets. L'injection répétée et lente de faibles doses (50 à 100 mcg/kg) nous a permis d'expérimenter.

Notons également que sur le diaphragme isolé de Rat, l'antagonisme par la néostigmine est plus marqué sur le venin de *N. nigricollis*.

VENINS D'*HYDROPHIIDAE*: *Lapemis hardwickii*, *Hydrophis cyanocinctus*, *Enhydrina schistosa*

(Venins provenant de l'Institut Pasteur de Saigon, Dr. Barme)

Les trois venins expérimentés ont des actions qualitatives communes, nous pouvons donc traiter leur action neuromusculaire en même temps.

TOXICITÉ — V. *Lapemis hardwickii*: DL_{50} i/v Souris 440 mcg/kg voisine de celle du *Naja haje*, le plus toxique des 3 *Naja* que nous avons étudiés. On constate une excitation légère après l'injection, après un temps de latence de

10 à 15 minutes, une gêne respiratoire s'installe, suivie d'une paralysie progressive généralement mortelle. Après une dose non mortelle, il faut au moins 5 heures pour que les troubles respiratoires disparaissent.

PRÉPARATION NEUROMUSCULAIRE DE RAT — L'excitation électrique indirecte par le nerf devient inefficace, l'excitation électrique directe par le muscle reste efficace (dose paralysante seuil 75-100 mcg/kg). Il y a une grande ressemblance avec l'effet du venin de *Naja*, mais ici pas d'effet hypotenseur conjoint gênant.

Donc type curarisation classique, mais la paralysie est irréversible. Néostigmine et édrophonium ont une action antagoniste fugace. Il y a sensibilisation nette aux doses non paralysantes de d-tubocurarine.

SUR LA PRÉPARATION ISOLÉE DIAPHRAGME DE RAT, on note une paralysie lentement irréversible s'installant après un temps de latence plus ou moins long selon la dose (2 mcg/ml — la paralysie apparaît après 20 à 30 min., devient totale, malgré les lavages, 30 à 40 minutes après). Là encore, l'excitation électrique indirecte par nerf devient inefficace, l'excitation électrique directe du muscle reste efficace. Mêmes antagonismes fugaces par néostigmine et édrophonium. Même sensibilisation nette aux doses non paralysantes de d-tubocurarine.

ACTION DE L'ACÉTYLCHOLINE EXOGÈNE

a) Sur un diaphragme chronique dénervé, isolé 10 jours après, l'excitation directe du muscle étant interrompue, l'addition d'A/C n'entraîne aucune contraction après venin d' HYDROPHIIDAE.

b) Sur une préparation *in situ* de Chat, l'excitation indirecte étant interrompue, l'injection dans l'artère tibiale d'A/C. n'entraîne aucune contraction après venin de *Lapemis* (Fig. 3).

RECTUS ABDOMINIS ISOLÉ DE GRENOUILLE

Sur ce test venins d' HYDROPHIIDAE: pas d'action propre, diminution considérable des effets de l'A/C. pas de sensibilisation aux ions K^+.

En conclusion action neuromusculaire du même type que celle des *Naja*, mais peut-être plus dépouillée. car il n'y a pas de facteur cardiovasculaire donc l'action hypotensive surajoutée rend parfois pour ceux-ci l'expérimentation difficile sur les préparations *in situ*.

Mécanisme d'action périphérique neuromusculaire des venins de Naja et d' HYDROPHIIDAE *étudiés.*

Le trois venins de *Naja* (*N. naja, N. haje, N. nigricollis*) et les trois venins d' HYDROPHIIDAE (*Lapemis hardwickii, Hydrophis cyanocinctus, Enhydrina schistosa*) étudies ont une action neuromusculaire du même type. Cette action ressemble nettement à une curarisation typique. On constate après leur administration:

Arguments pour type curarisation classique (type Claude Bernard)

1 — l'excitation électrique indirecte par le nerf devient inefficace, l'excitation électrique du muscle reste efficace.

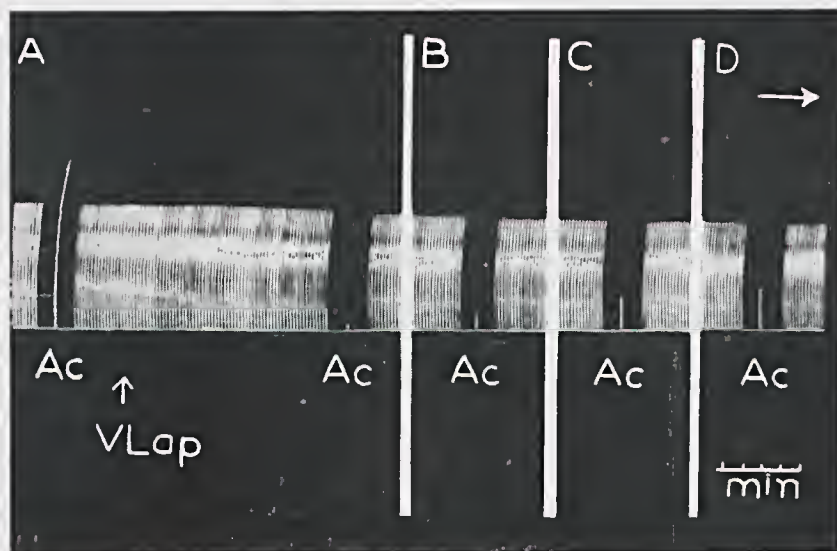



Fig. 3 — CHAT: Contractions du muscle tibial antérieur *in situ*. Stimulation du nerf sciatique (0,3 msec — 3 V — 0,1 c/sec) interrompue pendant 2 min pour permettre l'injection rapide d'acétylcholine en Ac (2,5 mcg) dans l'artère tibiale (portion distale à contre courant). A: contracture témoin en V Lap, injection i/v de 50 mcg/kg de venin de *Lapemis* (dose paralysante seuil). La contracture est presque totalement inhibée au bout de 12 min: elle récupère ensuite progressivement au bout de 30 min (B), 45 min (C) et 1 h (D), mais sans jamais pouvoir atteindre l'amplitude initiale.

2 — néostigmine, édrophonium, antagonisme, net mais fugace.

3 — synergie avec les acétylcholinocompétitifs, antagonisme (?) avec acétylcholinemimétiques.

4 — injection directe dans l'artère (Prep. nerf-muscle *in situ*) inefficace addition d'A/C diaphragme isolé innervé ou énervé (depuis 15 jours) inefficace.

Ceci montre que l'A/C exogène n'agit plus sur les récepteurs spécifiques et laisse supposer qu'il en est de même pour l'A/C endogène. S'il y a diminution de la synthèse de l'A/C (?), cela ne tient pas à un déficit en choline, l'administration de celle-ci ne modifiant pas la paralysie.

Aux doses supraliminaires de venin, l'augmentation de fréquence des excitations (augmentant la consommation d'A/C endogène) paraît accélérer la paralysie.

5 — diminution ou suppression des effets de l'A/C sur le *rectus abdominis* de Grenouille.

Arguments contre une curarisation classique:

1 — temps de latence plus ou moins prolongé et paralysie irréversible,

2 — pas d'antagonismes vrais, puisque fugaces.

Bien qu'il semble y avoir avec ces venins une action sur les récepteurs spécifiques à l'acétylcholine de la plaque motrice, on ne peut donc l'assimiler à la paralysie réversible par les acétylcholinocompétitifs (curares historiques ou substances de synthèse). Le blocage des récepteurs paraît ici irréversible.

Conclusions

D'une façon un peu simpliste, on peut opposer:

L'action *musculaire* des venins de CROTALIDAE: contracturante dans le cas des venins à crotamine, paralysante pour les venins sans crotamine.

L'action curarisante de type compétitif des venins de *Naja* et d'HYDROPHIIDAE.

Summary

I. The neuromuscular actions of several cobra (common cobra, *Naja naja*, Egyptian cobra, *N. haje*, and spitting cobra, *N. nigricollis*) and sea-snake (*Lapemis hardwickii*, *Hydrophis cyanocinctus* and *Enhydrina schistosa*) venoms were studied as well as those of both varieties of the South American rattlesnake (*Crotalus durissus terrificus*) venom, the crotamin containing one and the variety devoid of that substance. The actions of crotamin itself, a basic protein isolated by Moura Gonçalves, was also investigated.

II. All three cobra venoms studied are neuromuscular blocking agents: they exert a peripheral, slowly induced and irreversible action. The paralysis of neuromuscular transmission appears concurrently with a cardiovascular depression on *in situ* preparations or with a contracture on isolated preparations. *N. haje* venom is a more potent neuromuscular blocking agent than a cardiovascular depressing or contracture inducing substance while *N. nigricollis* venom is more active in producing cardiovascular depression or skeletal muscle contracture than in eliciting neuromuscular blockade. The venom of *N. naja* occupies, from this point of view, an intermediate position.

1. The skeletal muscle paralysis is not due to a direct action of the cobra venoms on the muscle fibre; the contracture, on the contrary, is produced at this level.

2. The interruption of neuromuscular transmission produced by the *Naja* venoms is, at least in some measure, explainable by a slow and irreversible inhibition of end-plate receptors: therefore their neuromuscular blocking action resemble a curarization in Cl. Bernard sense. It is with the venom of *N. naja* that this inhibition of receptors can be better evidenced.

3. It is assumed, without direct evidence, that these venoms also exert a presynaptic action.

III. All three HYDROPHIIDAE venoms studied show qualitatively identical neuromuscular blocking properties. Their mechanism of action is very close to that of the *Naja* venoms: paralysis of neuromuscular transmission by a slow

and irreversible inhibition of end-plate receptors. On the other hand, the HYDROPHIIDAE venoms, contrary to the cobra venoms, do not produce cardiovascular depression or contractures.

IV. The crotamin containing variety of rattlesnake venom and crotamin itself show contracture inducing properties on skeletal muscles which can be observed on intact and conscious animals as well as on *in situ* or isolated preparations. This effect is produced by a direct action of crotamin on muscle fibres. A secondary paralysis is inconstantly produced.

In spite of some similarities with veratrin, the action of crotamin and crotaminic venom can not be compared with a veratrinic one.

V. The non crotaminic rattlesnake venom shows a weak and slowly induced paralytic effect which can not be easily evidenced on account of its strong cardiovascular depressing properties.

This paralytic effect is due to a direct action on the muscle fibre without inhibition of the end-plate receptors.

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Discussion

A. Barrio comments: "Nos cupo a Oswaldo Vital Brazil y a mi señalar por primera vez la existencia de dos tipos de veneno de *Crotalus durissus terrificus* en cuanto a su acción neuromuscular: uno (tipo I) que producía paralisis y otro (tipo II) que en forma muy llamativa provocaba espasmos. Posteriormente pude demonstrar que este cuadro era producido por la crotamina, substancia aislada por Moura Gonçalves, del veneno de *C. durissus terrificus* procedente de la Argentina. Comparamos la acción del veneno tipo II con la veratrina. Aclaro que denominamos a esta ponzoña "veratrine-like", nunca quisimos señalar identidad absoluta y total entre estas dos substancias."

58. MODES OF ACTIONS OF PURIFIED TOXINS FROM ELAPID VENOMS ON NEUROMUSCULAR TRANSMISSION

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The actions of venoms from snakes belonging to the family of ELAPIDAE on neuromuscular transmission have recently been reviewed by Meldrum (1). The view that peripheral paralysis of respiratory muscles is the principal cause of death from elapid venoms appears to be well established (2, 3, 4). The curare-like action of elapid venoms has been extensively studied and several authors have presented evidence that neuromuscular blocking venoms have a postsynaptic site of action (for references see Meldrum (1)). Although a "non-depolarizing" block of neuromuscular transmission, like that of curare, has been postulated as the mode of action for Formosan cobra (*Naja naja atra*) venom (5) and banded krait (*Bungarus multiciuctus*) venom (6), some differences between actions of these venoms and those of curare were also noted. Thus, the neuromuscular block was not effectively relieved by anticholin-esterases or reversed by washing-out; no Wedensky inhibition was observed on repetitive indirect stimulation of the muscle paralysed by these venoms at higher concentrations; and acetylcholine release from the presynaptic terminals was more or less impaired. Besides, the action of cobra venom is complicated with a direct musculotropic effect (5), and a depolarizing activity on skeletal muscle has been found in this venom (7).

Since snake venom could be regarded as a mixture of proteins or polypeptides, it was considered that the complexity of the venom actions might be due to combined effects of different components contained in the same venom. Using zone electrophoresis on starch at pH 5.0, the venom of *B. multicinctus* was separated into four fractions (8). One lacks neuromuscular blocking properties but contains cholinesterase. One called "α-Bungarotoxin" produces a neuromuscular block of relatively rapid onset *in vitro* and *in vivo*. It does not alter the acetylcholine output from the rat phrenic nerve endings but it abolishes the response of the chick biventer cervicis muscle to acetylcholine. The two most electropositive fractions, called "β- and γ-Bungarotoxin" respectively, produce neuromuscular block and a severe reduction in acetylcholine output in the rat diaphragm after a latent period of about one hour and this period is not shortened by increasing the dose. Neuromuscular block produced by these two fractions in the chick biventer cervicis muscle is not associated with any diminution of sensitivity to acetylcholine. Mice given large doses of these fractions show hyperirritability at first and, after a latent period of about one hour, die suddenly with dyspnea and convulsions.

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From *Naja naja atra* venom two toxic fractions were separated under the same electrophoretic conditions (9, 10). One called *Najatoxin* or *cobra neurotoxin* produces *non-depolarizing* block of neuromuscular transmission but, unlike the whole venom, does not diminish the acetylcholine output from the rat phrenic nerve, nor has any direct action on the muscle fibres. This neurotoxin is also free from phospholipase A, histamine releasing and local irritant activities contained in the whole venom. Another fraction, which is the most electropositive and called *cardiotoxin*, produces a neuromuscular block with contracture, cardiac arrest in systolic state and many other pharmacological effects (11).

From the above-mentioned studies, it is obvious that at least two types of effects are observable at the neuromuscular junction. A curare-like non-depolarizing block is produced by cobra neurotoxin and α-Bungarotoxin. The other type of effect is impairment of acetylcholine release from the presynaptic terminals, which is produced by β- and γ-Bungarotoxins.

The present paper describes the results of our recent electrophysiological study of these purified toxins on the neuromuscular transmission. A preliminary account of some of the results has been given in a communication at the XXIII International Congress of Physiological Sciences (12).

Materials and methods

Purification of toxins: — α- and β-Bungarotoxins were isolated from the venom of *Bungarus multicinctus* according to the method described by Chang & Lee (8). Cobra neurotoxin and cardiotoxin were purified from the venom of *Naja naja atra* by CM-Sephadex column chromatography as described by Lo, Chen & Lee (13). Potencies of these purified toxins were checked by their toxicities in mice and effects on the chick biventer cervicis muscle.

Intracellular microelectrode recording: — The conventional microelectrode recording technique (14) was adopted using glass microelectrodes filled with 3M KCl and having 6-10 MΩ resistance. No capacity compensation for the microelectrode was incorporated. Grass model P6 DC preamplifier with its cathode-follower probe and Tektronix 502A oscilloscope were used. For the rat phrenic nerve-diaphragm preparation, Tyrode solution oxygenated with 95% O_2 + 5% CO_2 was employed. The temperature was kept at 32-35 ± 0.5°C. For the frog nerve-sartorius muscle the preparation was suspended in Ringer solution, containing NaCl 117 mM, KCl 2.0 mM, $CaCl_2$ 1.8 mM and $NaHCO_3$ 6 mM, at room temperature (20-24°C).

The end-plate focus was localized with the aid of the time-course of the miniature end-plate potential (EPP) or evoked EPP. Indirect stimulation was applied through a pair of electrodes on the nerve with supramaximal rectangular pulses of 0.2 msec duration, and direct stimulation through electrodes, one on the muscle-tendon junction and the other in the bath fluid, also with 0.2 msec rectangular pulses.

Terminal nerve spike: — Extracellular recording of the terminal nerve spike with a microelectrode having resistance of about 5 MΩ was performed on the frog sartorius muscle, according to the technique described by Katz & Miledi (15). The muscle was immobilized by adding 11 mM $MgCl_2$ to the Ringer solution. Under such condition, the terminal nerve spike potential could be recorded together with an EPP.

Antidromic activity: — The method described by Randić & Straughan (16) for the recording of antidromic activity of the isolated rat phrenic nerve was followed. To the modified Tyrode solution, containing 3.6 mM $CaCl_2$ and 0.12 mM $MgCl_2$, neostigmine methylsulphate was added to give a final concentration of 0.3 μg/ml. The temperature was kept at 22 ± 0.5°C. Under this condition it was possible to record the antidromic repetitive discharges of the nerve following single nerve volleys for more then 2 hours if the stimulus frequency was kept at 0.05/sec or less.

Biventer cervicis nerve-muscle preparation of the chick: — The isolated biventer cervicis nerve-muscle preparation (17) was suspended in 20 ml of Krebs solution which was maintained at 37 ± 0.5°C and bubbled with 95% O_2 and 5% CO_2. The preparation was stimulated indirectly with supramaximal rectangular pulses of 0.5 msec duration at a rate of 6 per min.

Results and discussion

Effect on resting membrane potentials: — As shown in Table I, all of the three purified neurotoxins, cobra neurotoxin, α- and β-Bungarotoxins, did not cause any changes in the resting membrane potentials of muscle fibres at either end-plate or non-end-plate zone of the rat diaphragm at a concentration as high as 10 μg/ml. In contrast, cardiotoxin as well as crude cobra venom caused a progressive reduction of the resting potentials. Our results are at variance with those of Meldrum (7) who reported that the neurotoxic fraction isolated from Indian cobra (*Naja naja*) venom depolarized the frog sartorius muscle. It is likely that Meldrum's neurotoxic fraction might be contaminated by the cardiotoxic component.

TABLE I — EFFECT ON RESTING MEMBRANE POTENTIALS

Membrane potentials were recorded from both the end-plate and non end-plate zones of muscle fibers of the rat diaphragm at the indicated periods after addition of 10 μg/ml of each agent. n = Number of observations.

TOXIN	Membrane potentials (mV ± S.D.)				
	Control	0-5 min	5-10 min	10-15 min	15-20 min
α-Bungarotoxin	75.3 ± 3.7 (n = 33)	77.3 ± 6.5 (n = 9)	79.3 ± 3.2 (n = 9)	81.6 ± 6.0 (n = 11)	74.7 ± 4.1 (n = 6)
β-Bungarotoxin	76.5 ± 5.9 (n = 27)	—	—	—	72.9 ± 8.2 (n = 23)
Cobra neurotoxin	78.1 ± 4.4 (n = 28)	81.4 ± 4.4 (n = 11)	77.0 ± 3.9 (n = 5)	78.4 ± 4.2 (n = 10)	76.0 ± 4.8 (n = 7)
Crude cobra venom	83.0 ± 3.7 (n = 30)	49.3 ± 8.3 (n = 9)	34.0 ± 17.2 (n = 11)	29.0 ± 6.2 (n = 9)	23.0 ± 6.8 (n = 8)
Cardiotoxin	81.4 ± 4.1 (n = 30)	73.7 ± 8.5 (n = 11)	54.4 ± 9.7 (n = 10)	45.3 ± 14.2 (n = 9)	28.9 ± 12.8 (n = 12)

Effect on action potentials: — As shown in Fig. 1, no appreciable changes both in the amplitude and time course were found in the action potentials elicited by direct stimulation of the muscle fibres paralysed by a high concentration (10 μg/ml) of either α-Bungarotoxin or cobra neurotoxin. This is in agreement with our previous findings that these neurotoxins do not affect the muscle fibre itself.
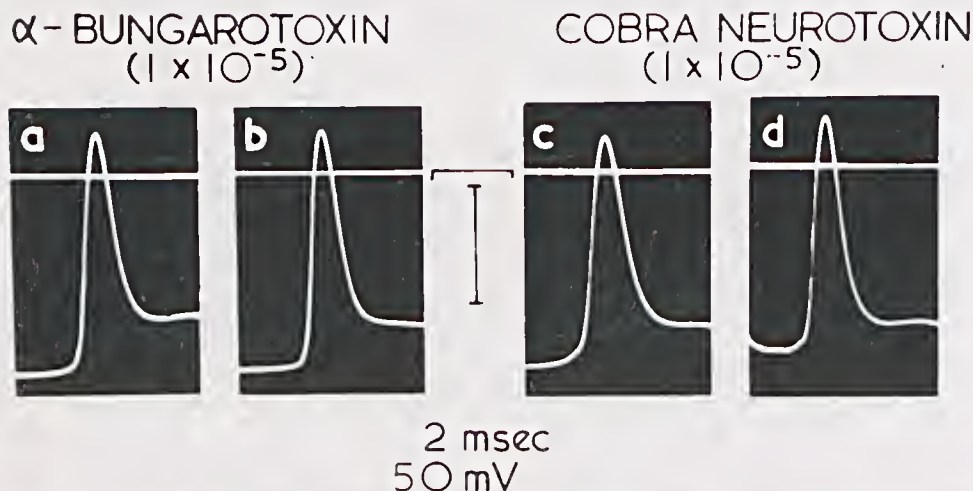
Fig. 1 — Effect on action potentials. Action potentials recorded from rat diaphragm preparations are shown. The first tracing of each pair (a & c) shows control action potential evoked by indirect stimulation. The second tracings are action potentials evoked by direct stimulation after the neuromuscular transmission was blocked by 10 μg/ml of α-Bungarotoxin (b) and 10 μg/ml of cobra neurotoxin (d), respectively.

Effect on end-plate potentials (EPPs): — EPPs were easily recorded from the muscle fibres of the rat diaphragm, immediately after their mechanical responses on nerve stimulation had been abolished by β-Bungarotoxin at a concentration of 1 μg/ml. By contrast, in the cases of α-Bungarotoxin and cobra neurotoxin, superficial muscle fibres were much more rapidly paralysed than the deeper ones and no EPPs could be recorded from the superficial muscle fibres when the muscle was paralysed. In order to record EPPs from the diaphragm paralysed by cobra neurotoxin, the preparations was first immersed in a concentration of 1 μg/ml for about one hour to block the neuromuscular transmission, then washed for one hour, and finally switched to a lower concentration, such as 0.03-0.05 μg/ml, which was just enough to prevent reappearance of mechanical response on nerve stimulation. Unlike cobra neurotoxin, the effect of α-Bungarotoxin was progressive and irreversible and hence no steady state could be attained in the presence of the toxin. In this case, lower concentrations, such as 0.4-0.5 μg/ml, were applied for about 2-3 hours and then, the preparation was washed with fresh Tyrode solution for recording of EPPs.

The time-courses of the EPPs thus obtained were compared with those obtained in the preparations paralysed by other neuromuscular blocking agents, such as dimethyltubocurarine (DMTC), decamethonium (C_{10}) and $MgCl_2$. As shown in Fig. 2 and Table II, the EPPs obtained in the diaphragm treated with each neurotoxin, whether acting presynaptically or postsynaptically, invariably showed a shorter time-course than those obtained in the muscle treated with
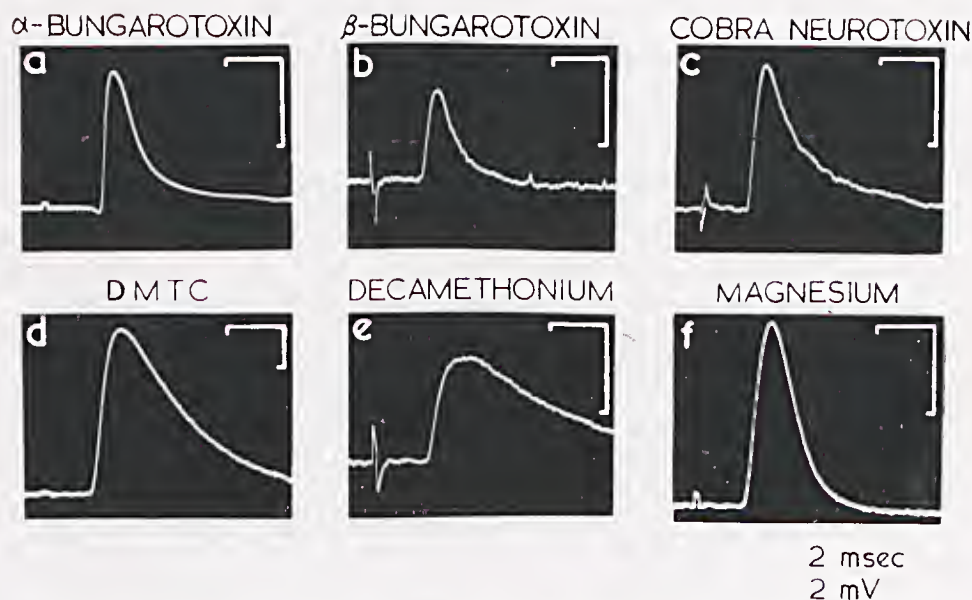
Fig. 2 — EPPs as affected by toxins and other neuromuscular blocking agents. EPPs recorded from the rat phrenic nerve-diaphragm preparation which was treated with the respective neuromuscular blocking agent as described in Table II.

TABLE II — TIME-COURSES OF THE EPPs AS AFFECTED BY NEUROTOXINS AND OTHER NEUROMUSCULAR BLOCKING AGENTS

The concentration of each agent shown in the table is the final one except that of α-Bungarotoxin and cobra neurotoxin; for details see the text. * For spontaneous miniature EPPs only those which had amplitudes comparable with the EPPs were selected. n = Number of observations.

AGENT	Concentration	n	Amplitude (mV±S.D.)	Time from onset to peak (msec±S.D.)	Time from peak to ½ decay (msec±S.D.)
α-Bungarotoxin	0.4 μg/ml for 3 hrs., then wash-out	19	2.35 ± 1.28	0.71 ± 0.14	0.62 ± 0.18
β-Bungarotoxin	1.0 μg/ml	17	3.04 ± 1.12	0.57 ± 0.11	0.97 ± 0.21
Cobra neurotoxin	1.0 μg/ml for 1 hr., then reduced to 0.05 μg/ml	68	3.14 ± 1.29	0.59 ± 0.13	1.02 ± 0.29
Dimethyltubocurarine	0.8 μg/ml	31	3.43 ± 0.94	0.82 ± 0.20	1.54 ± 0.12
Decamethonium	40.0 μg/ml	71	3.41 ± 1.16	0.95 ± 0.28	2.38 ± 0.78
Mg^{++}	12 mM	45	3.04 ± 0.60	0.41 ± 0.03	0.71 ± 0.09
Miniature EPP*	—	21	2.27 ± 0.46	—	0.78 ± 0.16

either DMTC or C_{10} and were rather close to those of the magnesium-paralysed muscle. These results suggest that the more prolonged EPP obtained in the muscle treated either with DMTC or C_{10} as compared with that obtained in the magnesium- or neurotoxin-paralysed muscle may be due to some pharmacological effect exerted by DMTC or C_{10} on the end-plate.

Effect on spontaneous miniature EPPs: — Brooks (18) has observed a decline in the frequency of miniature EPPs in a preparation treated with botulinum-toxin. Neuromuscular block occurred either before or after the complete disappearance of miniature EPPs. This result led him to conclude that botulinum-toxin blocks the neuromuscular transmission at the nerve terminals rather than at the arborization as postulated before by himself (19). The effect of β-Bungarotoxin was studied in the conjunction since it also blocks neuromuscular transmission at presynaptic site (8). The frequency of appearance of miniature EPPs was first increased 2-3 times by β-Bungarotoxin (0.3-3 μg/ml) during 1-2 hours after the application, then gradually decreased and finally no miniature EPPs could be found (Figs. 3 & 4). Stimulation of the phrenic nerve revealed that EPPs were abolished before the complete disappearance of miniature EPPs. It was occasionally found that a burst of miniature EPPs appeared for several minutes before their complete disappearance (Fig. 5).

In contrast, both α-Bungarotoxin and cobra neurotoxin reduced the amplitude of miniature EPPs progressively without affecting the rate of discharge, and finally the miniature EPPs had disappeared before neuromuscular block took place.
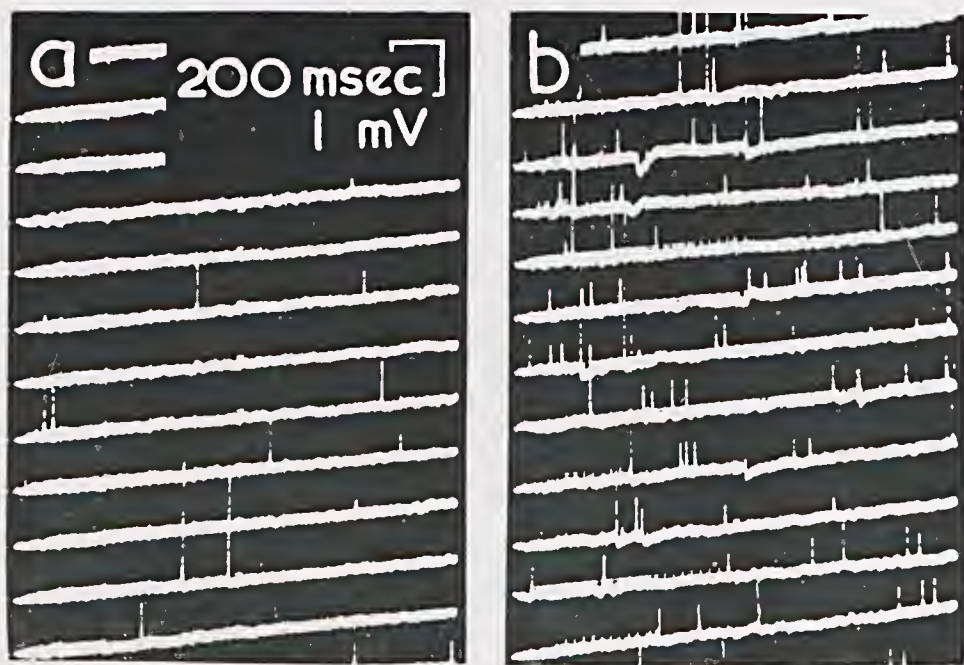
Fig. 3 — Effect of β-Bungarotoxin on the spontaneous miniature EPPs. Continuous recording on moving film, 2 sec per sweep. (a) Control recorded 45 min after setting up of the preparation; (b) 80 min after addition of 0.3 μg/ml β-Bungarotoxin. Note the increase in the frequency of miniature EPPs.
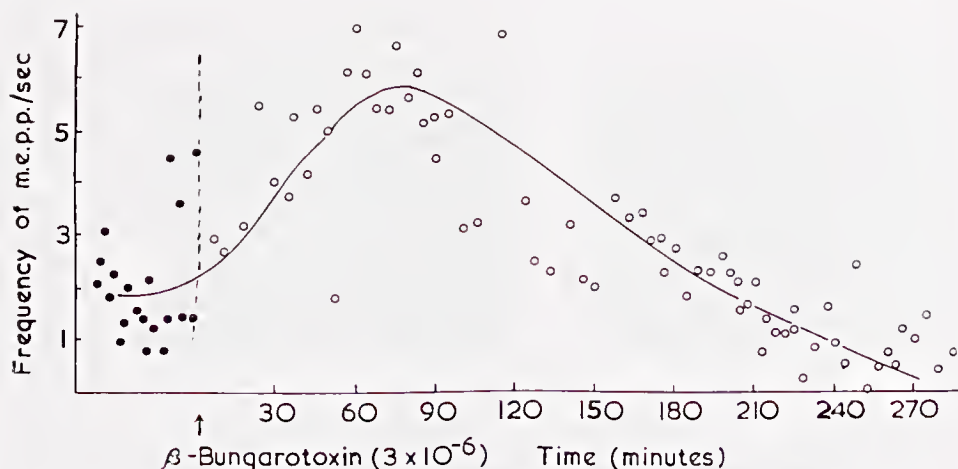
Fig. 4 — Effect of β-Bungarotoxin on the frequency of miniature EPPs. A different preparation from that shown in Fig. 3. Control was taken 30-60 min after setting up of the preparation and shown as the filled circle in the left side. β-Bungarotoxin (3 μg/ml) was added at 0 time. At 240 min, none of muscle fibres contracted on nerve stimulation. Each circle represents the mean frequency of miniature EPPs obtained from 20 sec observation of one end-plate.
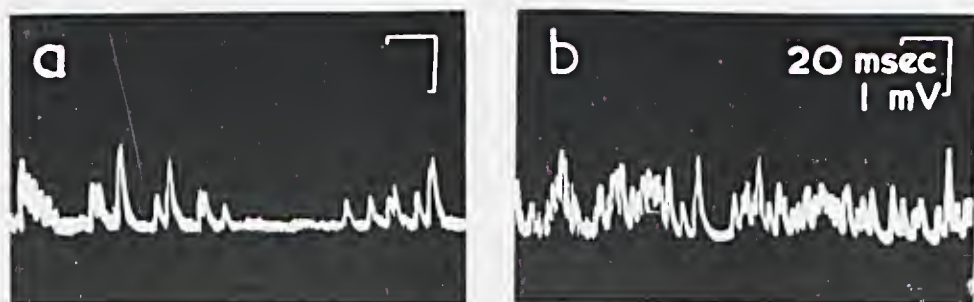
Fig. 5 — Burst of miniature EPPs induced by β-Bungarotoxin. The same preparation as described in Fig. 3. A burst of miniature EPPs from the same end-plate is shown. It was recorded at about 200 min after addition of 0.3 μg/ml β-Bungarotoxin. There was about 1 min interval between (a) and (b). This end-plate did not respond to nerve stimulation when the record was taken.

Antagonism with neostigmine: — As shown in Fig. 6 (a & b), in the rat diaphragm, treated with either α-Bungarotoxin or cobra neurotoxin, the EPP was increased in its size and prolonged in its time-course by neostigmine (1 μg/ml). Some of the EPPs became large enough to generate action potentials. Neostigmine also exerted a similar effect on the miniature EPPs recorded in the diaphragm after the evoked EPPs had been abolished by β-Bungarotoxin (Fig. 6, c & d). It must be stated here, however, that α-Bungarotoxin blocks the neuromuscular transmission more rapidly in the presence of anticholinesterase agents (8).
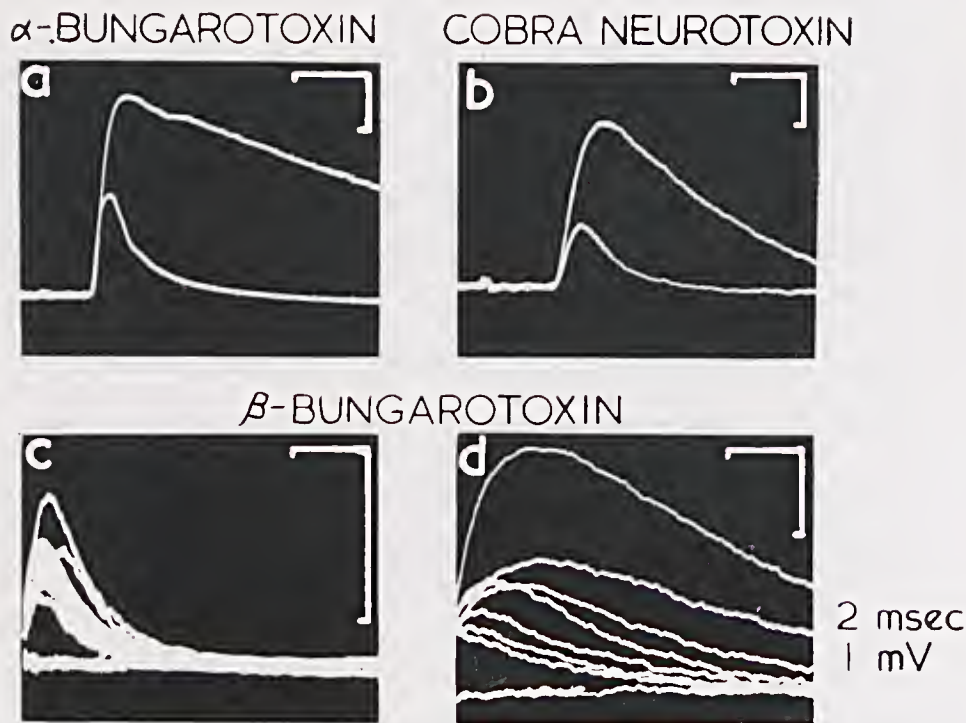
Fig. 6 — Effect of neostigmine on EPPs. Rat phrenic nerve-diaphragm preparations were respectively treated with the three neurotoxins as described in Table II. In a & b, lower tracing shows EPP before, and upper tracing 20 min after addition of 1 μg/ml of neostigmine in diaphragm paralysed by α-Bungarotoxin (a) and cobra neurotoxin (b), respectively. In c & d, it is shown superimposed miniature EPPs before (c) and after addition of neostigmine (d) in the β-Bungarotoxin-treated diapraghm.

Effect on the train of EPPs: — It has been shown that the rat diaphragm preparation treated with α-Bungarotoxin responds with a sustained contraction to repetitive nerve stimulation, whereas cobra neurotoxin (10) and the presynaptical-ly-acting β-Bungarotoxin (8) behave just like d-tubocurarine, causing Wedensky inhibition. On the other hand, botulinum-toxin, another presynaptic poison, does not show Wedensky inhibition in the guinea-pig diaphragm preparation (20). It is generally considered that the rapid decline of the successive EPPs in response to repetitive stimulation is the cause of failure to get a sustained contraction. Inability of the muscle to maintain the height of EPPs on repetitive stimulation has been interpreted to be due to a presynaptic phenomenon (21) or alternatively to desensitization of the postsynaptic membrane to acetylcholine (22). In order to gain more insight into the mode of action of these neurotoxins, trains of EPPs were elicited with successive pulses at various intervals in the rat diaphragm blocked by each neurotoxin and compared with those obtained in the muscle paralysed by other blocking agents. The EPP sequences obtained with pulse interval of 10 msec are shown in Fig. 7. It is apparent that EPPs declined at different rates from one agent to another. For the sake of comparison, the amplitude of successive EPPs was calculated as per cent of the amplitude of the first EPP (Table III). The rate of decline of EPPs and its relation to the

TABLE III — THE TRAIN OF EPPs AS AFFECTED BY POSTSYNAPTIC BLOCKING AGENTS

Phrenic nerve was stimulated with a train of supramaximal pulses for 100 msec. at various pulse intervals. Amplitude of the second, fifth and tenth EPP was expressed as per cent of that of the first EPP, respeetively. n = number of observations.

Agent	Pulse intervals (msec)	n	E.P.P. 1st (mV±S.D.)	2nd (% ± S.D.)	5th (% ± S.D.)	10th (% ± S.D.)
Dimethyltubocurarine, (0.5-0.8 µg/ml)	50	27	4.95 ± 2.04	73.9 ± 16.4		
	20	24	4.50 ± 2.08	79.2 ± 19.7	45.6 ± 15.5	
	10	37	4.38 ± 1.56	83.2 ± 19.7	48.1 ± 19.6	28.5 ± 10.3
	5	24	3.72 ± 1.22	92.4 ± 22.9	50.8 ± 22.2	30.0 ± 13.1
Decamethonium 50 µg/ml	50	36	2.01 ± 0.96	50.8 ± 0.84		
	20	36	2.01 ± 0.84	47.9 ± 7.2	28.7 ± 6.6	
	10	34	2.09 ± 0.85	46.1 ± 7.6	25.1 ± 6.4	24.7 ± 5.8
	5	7	2.10 ± 0.97	47.6 ± 6.3	22.4 ± 7.4	16.4 ± 4.5
Cobra neurotoxin 1 µg/ml for 1 hr. then reduced to 0.03-0.08 µg/ml	50	76	2.36 ± 1.22	74.1 ± 15.7		
	20	72	2.28 ± 1.10	84.2 ± 21.2	50.6 ± 18.7	
	10	75	2.34 ± 1.38	89.2 ± 26.8	56.9 ± 25.1	39.1 ± 22.5
	5	48	2.22 ± 0.97	100.9 ± 24.9	61.6 ± 25.6	41.8 ± 18.7
α-Bungarotoxin 0.5 µg/ml for 3 hrs. then wash-out	50	29	3.86 ± 1.72	84.4 ± 12.9		
	20	26	3.17 ± 1.32	93.7 ± 16.0	71.4 ± 19.3	
	10	29	3.76 ± 1.19	106.0 ± 23.9	70.7 ± 24.8	47.6 ± 16.2
	5	11	3.56 ± 0.70	98.4 ± 16.9	77.5 ± 11.6	57.6 ± 17.3

pulse intervals in the DMTC-treated muscle were similar to those obtained by Hubbard (23). To our surprise, however, C_{10} elicited a much more rapid decline of EPPs in the rat diaphragm preparation and caused a marked Wedensky inhibition, contrary to the finding that C_{10} caused a sustained contraction in the cat tibialis muscle (24).

The EPPs in cobra neurotoxin-treated preparation declined almost as rapidly as those in DMTC-treated one. On the other hand, the EPPs in the preparation treated with α-Bungarotoxin showed much slower decline than did the EPPs in preparations treated with other blocking agents described above. Actually in some junctions the EPPs maintained a constant level (Fig. 7, a).
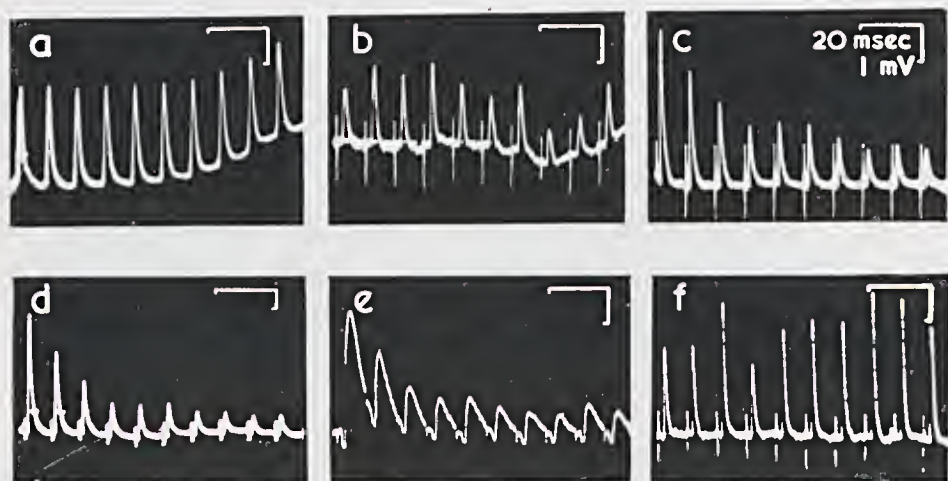
Fig. 7 — Patterns of trains of EPPs. The phrenic nerve was stimulated with a train of 10 pulses at the frequency of 100/sec. The experimental conditions were the same as described in Table II. (a) α-Bungarotoxin; (b) β-Bungarotoxin; (c) cobra neurotoxin; (d) Dimethyltubocurarine; (e) Decamethonium; (f) 12 mM $MgCl_2$.

In contrast to these agents, all of which are believed to act on the post-synaptic membrane, β-Bungarotoxin caused the amplitude of EPPs to fluctuate irregularly (Fig. 7, b), and there was occasional conduction block so that some EPPs were left out. Analysis of the EPP sequence revealed that no decline in its amplitude occurred during the train of 10 pulses (Table IV). As shown in Table IV and Fig. 7 f, treatment with high magnesium ion also caused a fluctuation of EPPs and facilitation as reported by del Castillo & Katz (25) and Hubbard (23) but no conduction block was observed. It is interesting in this regard that both β-Bungarotoxin and magnesium ion block the neuromuscular transmission by inhibition of acetylcholine release.

TABLE IV — THE TRAIN OF EPPs AS AFFECTED BY PRESYNAPTIC BLOCKING AGENTS

Phrenic nerve was stimulated with a train of supramaximal pulses for 100 msec at 10 msec pulse interval. Amplitude of the EPP was shown as the mean (mV ± S.D.) since the EPP fluctuated irregularly (Fig. 7-b) and had no definite correlation with the first one.

AGENT	n	E.P.P. (mV ± S.D.)			
		1st (mV ± S.D.)	2nd (mV ± S.D.)	5th (mV ± S.D.)	10th (mV ± S.D.)
β-Bungarotoxin 1×10^{-6} for 3 hours, then wash-out	13	1.47 ± 0.74	1.62 ± 0.80	1.48 ± 0.71	1.35 ± 0.72
Mg, 12 mM	8	2.44 ± 1.10	3.14 ± 0.83	3.38 ± 0.97	3.76 ± 1.16

However, conduction block, occurred in the β-Bungarotoxin-treated muscle during repetitive stimulation, became more marked and the average amplitude of EPPs was reduced as the stimulation was prolonged. As shown in Fig. 8 and Table V, the conduction failure increased to more than twice during 500 msec and the average amplitude of EPP decreased by about 66%. In contrast, neither conduction block nor decrease in the amplitude of EPP was observed in the magnesium-paralysed muscle during prolonged stimulation. These results may explain why the muscle treated with β-Bungarotoxin tends to show Wedensky inhibition.
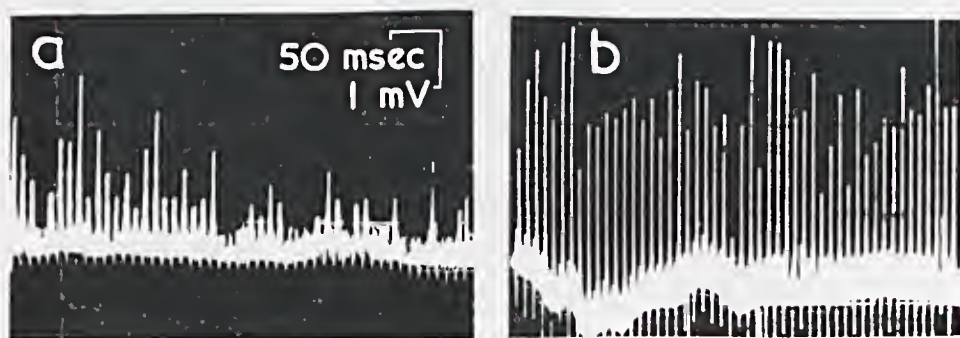
Fig. 8 — Effect of β-Bungarotoxin and Mg ion on the train of EPPs. The phrenic nerve was stimulated with a train of 50 pulses at the frequency of 100/sec. (a) Treated with 1 μg/ml β-Bungarotoxin for 185 min and then washed. Note the failure in response to the stimulation and decrease in the amplitude of EPP. (b) 12 mM $MgCl_2$.

TABLE V — COMPARISON OF THE RATE OF FIRING AND THE AMPLITUDE OF FIRED EPP ON REPETITIVE STIMULATION BETWEEN PREPARATIONS TREATED WITH β-BUNGAROTOXIN AND THOSE WITH Mg ION

The diaphragm was treated either with 1 μg/ml β-Bungarotoxin or with 12 mM Mg-Tyrode's solution. A train of 50 pulses at the frequency of 100/sec was given. The number of responses to the first 10 pulses and that to the last 10 pulses of the train were counted and the average amplitude of EPPs was compared.

AGENT	n	No. of junctions fired (mean ± S.D.)		Amplitude of EPP (mV ± S.D.)	
		First 10 stimuli	Last 10 stimuli	First 10 stimuli	Last 10 stimuli
Mg^{++}	8	10 ± 0	10 ± 0	3.66 ± 1.06	3.35 ± 1.08
β-Bungarotoxin	13	7.5 ± 0.5	3.5 ± 0.5	1.10 ± 0.76	0.37 ± 0.32

Effect on antidromic activities: — It has been shown that curare abolishes repetitive antidromic discharges of motor nerve fibres, evoked by orthodromic nerve impulse under the influence of anticholinesterase agents, at a dose level much lower than that required for blocking neuromuscular transmission (26, 27). As shown in Fig. 9, the repetitive discharges of the rat phrenic nerve evoked

by a single volley in the presence of neostigmine (0.3 μg/ml) were abolished by all of the three neurotoxins before the occurrence of complete neuromuscular block.
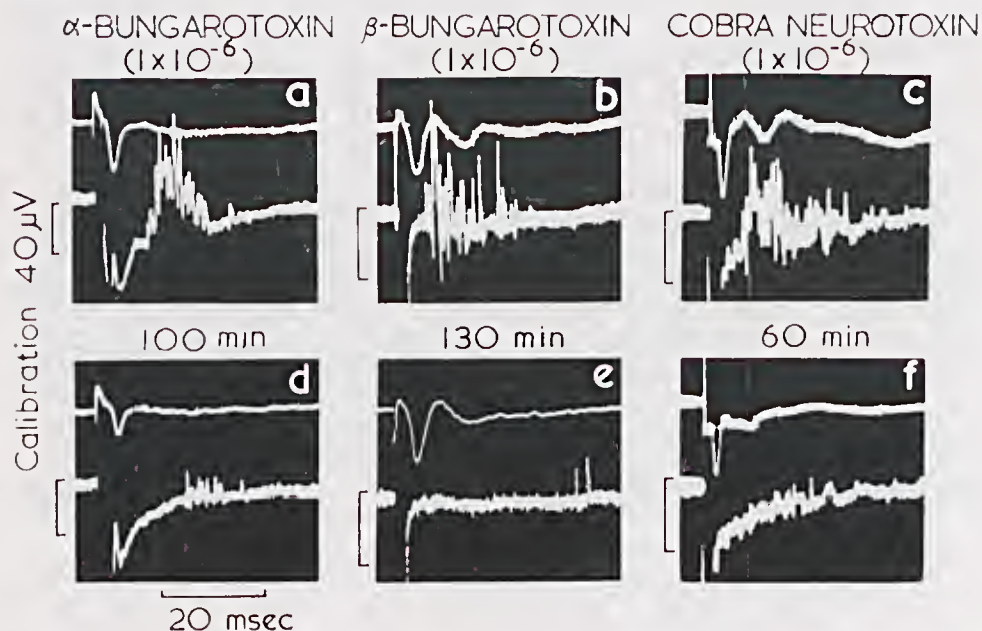
Fig. 9 — Effect on antidromic activities. Lower tracing in each figure shows the antidromic activities of the rat phrenic nerve preparation in the presence of neostigmine (0.3 μg/ml) as described in the METHOD. Upper tracing shows the muscle action potential monitored with an extracellular microelectrode. Upper figures (a, b & c) show the control activities and lower figures, (d) 100 min after α-Bungarotoxin (1 μg/ml), (e) 130 min after β-Bungarotoxin (1 μg/ml) and (f) 60 min after cobra neurotoxin (1 μg/ml), respectively.

Effect on terminal nerve spike: — While the terminal nerve spike recorded with extracellular microelectrode in the frog sartorius was abolished by cardiotoxin (Fig. 10), it remained unaffected after the EPP, simultaneously recorded with the same electrode, had been abolished by each of the three neurotoxins (Fig. 11). These findings indicate that none of these neurotoxins interferes with the conduction in nerve axons up to the nerve terminals, whereas cardiotoxin blocks nerve conduction probably by its depolarizing effect. This effect of cardiotoxin may explain the inhibition of acetylcholine output from the nerve endings by the crude cobra venom (10). On the other hand, since the conduction in nerve axons is unaffected by β-Bungarotoxin up to the nerve terminals, the inhibition of acetylcholine release by this toxin is probably due to its action on the excitation-secretion coupling system.

Effect of stimulus frequency on the time-course of neuromuscular block by β-Bungarotoxin: — Hughes and Whaler (28) have recently shown that nerve stimulation with higher frequencies causes a marked enhancement of neuromuscular block by botulinum-toxin. This phenomenon was interpreted as produced by an increase of the permeability to the botulinum toxin in consequence of a sustained depolarization of the nerve endings caused by nerve stimulation at
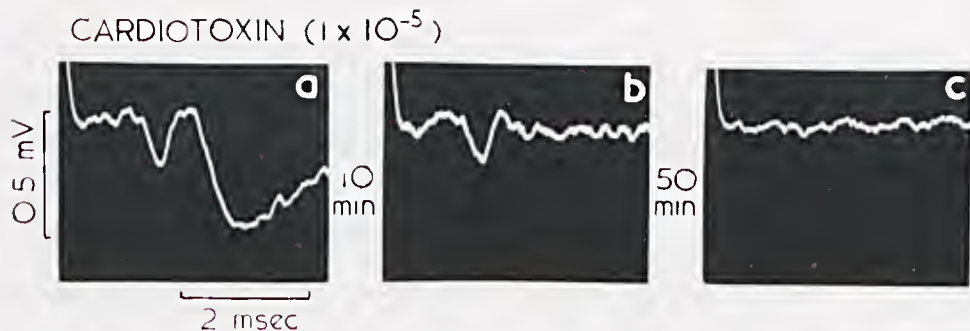
Fig. 10 — Effect of cardiotoxin on terminal nerve spike. The frog nerve-sartorius preparation immobilized with 11 mM $MgCl_2$. The first figure (a) shows a control terminal nerve spike with an accompanied EPP. The second (b) and the third (c) were recorded at 10 min and 50 min after addition of cardiotoxin (10 μg/ml), respectively.
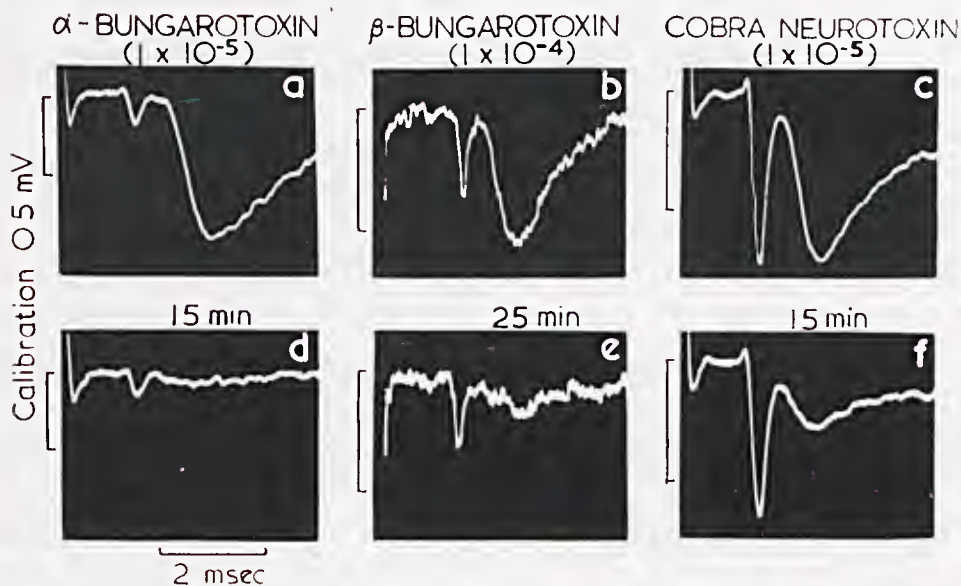
Fig. 11 — Effect of the neurotoxins on terminal nerve spike. Frog nerve sartorius preparations immobilized with 11 mM $MgCl_2$. The upper figures (a, b & c) respectively show a control terminal nerve spike with an accompanied EPP. The lower figures show the effects of respective neurotoxins: (d) 15 min after α-Bungarotoxin (10 μg/ml); (e) 25 min after β-Bungarotoxin (100 μg/ml); and (f) 15 min after cobra neurotoxin (10 μg/ml). Note the high concentration used for β-Bungarotoxin because of low sensibility of the frog sartorius muscle to this neurotoxin.

higher frequencies. It seemed to be interesting, therefore, to see whether the action of β-Bungarotoxin, a presynaptic poison, would be similarly affected. The stimulus frequency was changed from 0.75/min. to 48/min. and the time necessary to cause complete neuromuscular block was recorded. Fig. 12 shows that as the frequency increased the time necessary for neuromuscular block was shortened. Lowering of the frequency below 3/min., however, did not further slow down the block. In contrast, neuromuscular block by α-Bungarotoxin was

scarcely affected by increasing the rate of stimulation. Since the uptake of β-Bungarotoxin appears to be a rather rapid process (8), it is unlikely that the enhancement of neuromuscular block is due to an increase in permeability to β-Bungarotoxin, caused by stimuli of high frequencies as postulated for botulinum-toxin by Hughes and Whaler (28). This result rather indicates that the action of β-Bungarotoxin on the nerve endings is influenced by the activity of the nerve endings.
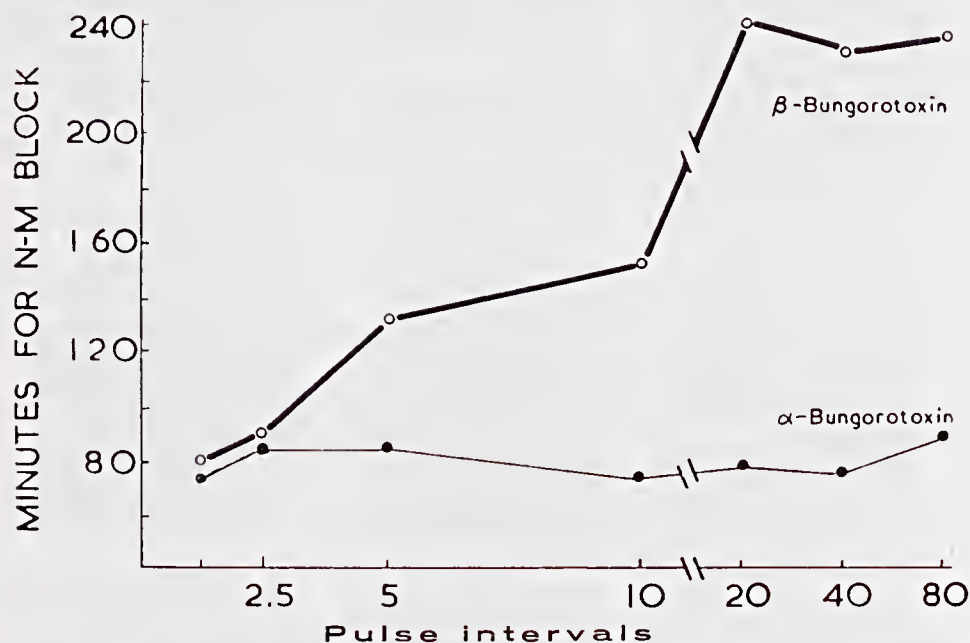
Fig. 12 — Effect of stimulus frequency on the time-course of neuromuscular block. The phrenic nerve was stimulated with single pulses at frequencies varying from 0.75/min to 48/min. Each point represents the mean of 4-15 experiments.

Effect of calcium and magnesium ions on the time-course of neuromuscular block by β-Bungarotoxin: — One hemidiaphragm preparation was immersed in modified Tyrode solution containing either low calcium (0.45 mM) or high magnesium (12 mM). The preparation usually failed to respond to nerve stimulation within 20 min. and β-Bungarotoxin (1 μg/ml) was then added. Interesting enough, the response to nerve stimulation was partially restored on addition of β-Bungarotoxin and then decreased progressively (Fig. 13). The contralateral hemidiaphragm was immersed in normal Tyrode solution and similarly treated with β-Bungarotoxin to serve as the control. When β-Bungarotoxin induced a complete neuromuscular block of the control preparation, both of the preparations were washed out with normal Tyrode solution. While no recovery of response occurred in the control preparation, the response of the test preparation was restored after repeated washing, and it took another 100-150 min. to cause neuromuscular block again. Thus, low calcium as well as high magnesium can markedly prolong the time for neuromuscular block by β-Bungarotoxin (Table VI). Such antagonism between β-Bungarotoxin and calcium or magnesium ion may suggest that they share a common site of action at the nerve endings.
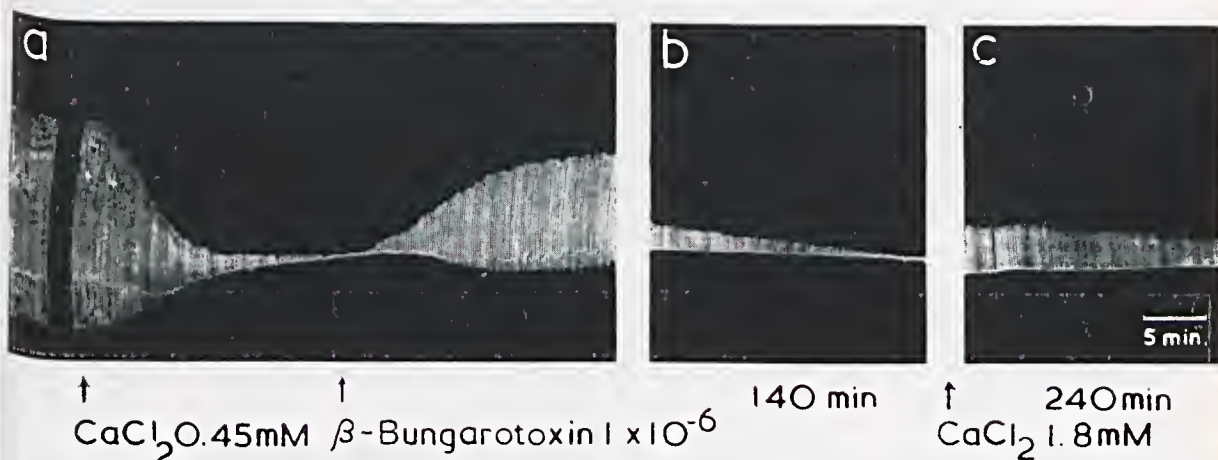
Fig. 13 — Effect of calcium and β-Bungarotoxin on neuromuscular transmission. Rat diaphragm preparation; for detail see the text. Note the restoration of neuromuscular transmission on addition of β-Bungarotoxin 1 μg/ml to the paralysed preparation.

TABLE VI — EFFECT OF CALCIUM AND MAGNESIUM ON THE NEUROMUSCULAR BLOCKING ACTION OF β-BUNGAROTOXIN

For the experimental detail see the text. The time taken for the test preparation to cause neuromuscular block was that from the addition of β-Bungarotoxin (1 μg/ml) to complete arrest after washing. n = no. of experiments.

Pretreatment	Concentration	Time for N-M block by β-Bungarotoxin (Min. ± S.D.)
None	—	153 ± 12.8 (n = 15)
$MgCl_2$	12.00 mM	266 ± 63.3 (n = 6)
$CaCl_2$	0.45 mM	313 ± 45.0 (n = 4)

Effect of d-tubocurarine pretreatment on the neuromuscular blocking action of α-Bungarotoxin: — It has recently been demonstrated that pretreatment with d-tubocurarine protects the chick biventer cervicis muscle from the neuromuscular blocking action of cobra neurotoxin (10). In order to see whether d-tubocurarine also can protect the muscle from the paralytic effect of β-Bungarotoxin, one of the two biventer cervicis muscles from the same chick was pretreated with d-tubocurarine (10 μg/ml) for 10 min. and the other was immersed in Krebs solution to serve as the control. α-Bungarotoxin (0.3 μg/ml) was added to both preparations simultaneously. When the response of the control preparation to nerve stimulation was abolished, both preparations were washed with fresh Krebs solution once every 5 min. As shown in Fig. 14, while no recovery was
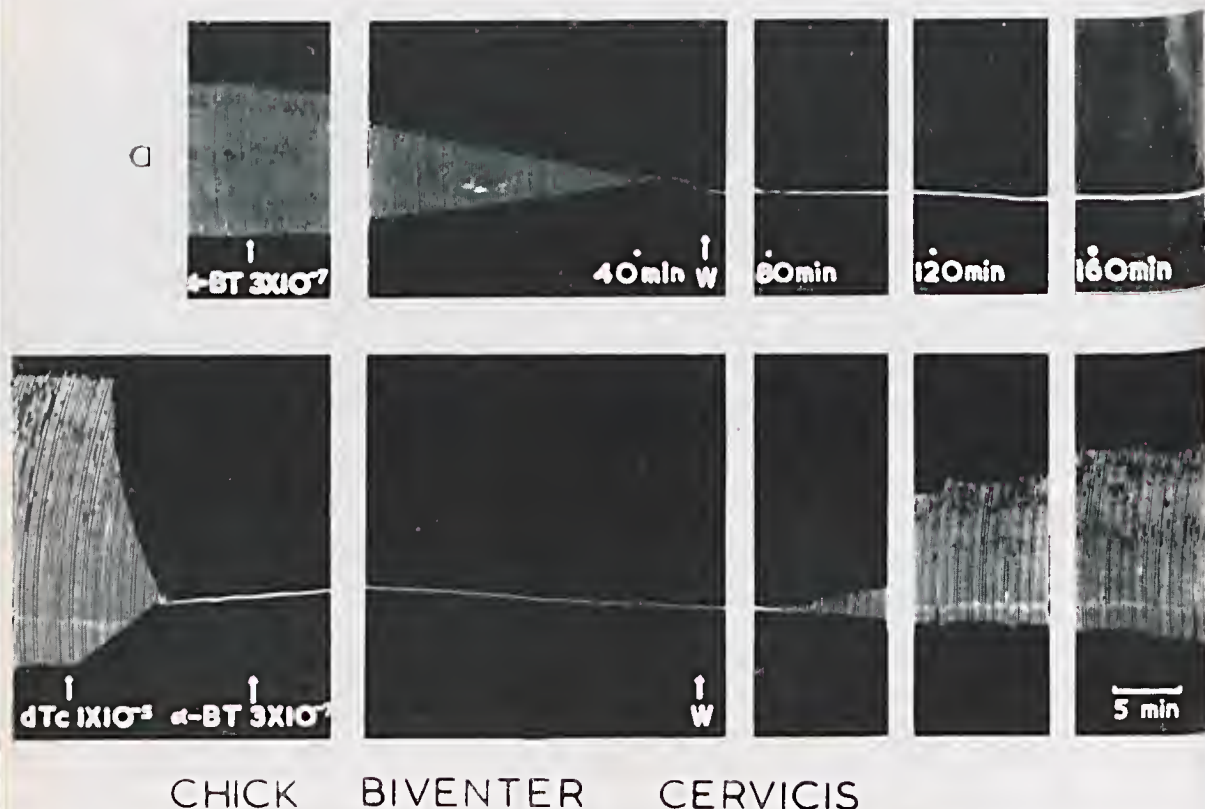
Fig. 14 — Effect of d-tubocurarine on the neuromuscular blocking action of α-Bungarotoxin. Chick biventer cervicis muscles. a: control treated only with α-Bungarotoxin, 0.3 μg/ml. b: contralateral preparation pretreated with 10 μg/ml of d-tubocurarine. Note the remarkable restoration of response after repetitive washing in the d-tubocurarine-pretreated preparation in contrast to the control.

found in the control preparation despite repeated washings for 3 hours, the response of the preparation pretreated with d-tubocurarine recovered steadily to about 70% of the original height after 3 hours' washing. In contrast, no protection by d-tubocurarine was found against the neuromuscular blocking action of β-Bungarotoxin. These findings, together with our previous observation with cobra neurotoxin, strongly suggest that α-Bungarotoxin as well as cobra neurotoxin blocks neuromuscular transmission by combining with the specific receptor of acethylcholine at the end-plate, just like d-tubocurarine does.

Summary and conclusions

Cobra neurotoxin: — Cobra neurotoxin depresses EPP without affecting resting membrane potential, muscle action potential, and terminal nerve spike. The amplitude of the EPP is increased and its time-course prolonged by neostigmine. Antidromic activities of the phrenic nerve in the presence of neostigmine are abolished. The amplitude of successive EPPs on repetitive stimulation declines markedly as in the curarized muscle. From these findings it is concluded that

the mode of neuromuscular blocking action of cobra neurotoxin is essentially similar to that of curare, although the former acts much more slowly and less reversibly than the latter.

α-Bungarotoxin: — The effects of α-Bungarotoxin on the neuromuscular transmission are similar to those of cobra neurotoxin in the following aspects: (1) depression of EPP without affecting resting membrane potential, muscle action potential and terminal nerve spike; (2) enlargement and prolongation of EPP by neostigmine; (3) inhibition of antidromic discharges of the motor nerve; and (4) protection from its paralytic effect by d-tubocurarine. However, α-Bungarotoxin differs from cobra neurotoxin in that its paralytic effect is irreversible and not restored by neostigmine and that the decline of successive EPPs is much less marked, so that sustained contraction is observed on repetitive stimulation.

β-Bungarotoxin: — β-Bungarotoxin increases the frequency of spontaneous miniature EPPs in the early stage and restores neuromuscular transmission impaired by either low calcium or high magnesium, but in the later stage, the number of miniature EPPs is diminished without change in size. Neuromuscular block as well as abolition of EPP takes place before the complete disappearance of miniature EPPs. All these findings are in good accordance with our previous conclusion that β-Bungarotoxin acts presynaptically, reducing the acethylcholine output from the nerve endings and leaving the sensitivity of the end-plate to acetylcholine unaffected. Since the terminal nerve spike remains unaffected, it appears that β-Bungarotoxin acts on the motor nerve endings, probably affecting the excitation-secretion coupling system.

Cardiotoxin: — Cardiotoxin causes contracture of the muscle as well as neuromuscular block by depolarizing both the muscle and nerve fibres; the terminal nerve spike is abolished and direct stimulation of the muscle fibre fails to evoke an action potential. This effect of cardiotoxin on nerve tissue is probably responsible for the reduction of acetylcholine output from the nerve endings caused by crude cobra venom.

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Discussion

F. E. Russell: "First, may I compliment you on a very interesting and definitive study. It is perhaps infortunate that curare was studied in depth before venoms, for we have a tendency to drift into the habit of calling things "curare-like". In reality, many substances have a "curare-like" activity, and these substances are in no way related chemically, and indeed the difficulties may not be as closely associated with chemistry as they are with the physiological abuse of "curare-like". Your work indicates that the common acceptance of certain venoms and their fractions as post-synaptic agents should be reexamined. I agree with you, and I would like to ask whether or not you care to discuss the specific site of action involved or if you have conducted studies in this direction?"

C. Y. Lee: "Although cobra neurotoxin as well as alpha-Bungarotoxin has some presynaptic action (e.g. such of antidromic discharges of motor nerve), we believe, N-M block caused by these two neurotoxins is post-synaptic and the specific site of action is acetylcholine receptor on the end-plate, since d-tubocurarine pretreatment can protect the paralysing action of these neurotoxins. On the other hand, the action of beta-Bungarotoxin is exclusively presynaptic, leaving the sensitivity of end-plate to acetylcholine unaffected. The site of action is on motor nerve endings, probably on the excitation-secretion coupling system, since the terminal nerve spike remains unaffected."

E. Garcia Mendes: "How about the molecular weight of the alpha- and beta-Bungarotoxin?"

C. Y. Lee: "The molecular weight of alpha-Bungarotoxin has been estimated to be about 8,000. Since beta-Bungarotoxin has not been obtained in pure state, the exact molecular weight is unknown, but we have evidence that it is higher than that of alpha-Bungarotoxin."

D. Mebs: "Is your *Naja*-neurotoxin-preparation the same of Mr. Yang? Is it free of enzymes?"

C. Y. Lee: "Judging from the LD50 in mice, our cobra neurotoxin is almost identical with Dr. Yang's cobratoxin and it is free from various enzymic activities, such as protease, cholinesterase, phospholipase A and hyaluronidase (unpublished observations)."

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59. CARDIOTOXIC AND CARDIOSTIMULATING FACTORS IN COBRA VENOM

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The subject of snakes and their venoms is of special interest to people of all tropical countries, as the number of deaths that occur every year all over the world runs, according to the World Health Organization is as high as 40,000. In India alone, some 12,000 to 15,000 people die every year and in South America 3,000 to 4,000 deaths occur each year. The number of deaths from snake bites is likewise considerably high compared to their total population; in Burma, Indo-China, Australia, Africa, West Indies, and the tropical belt of North and South America. The temperate parts of the globe are far less severely affected.

The venoms of snakes belonging to ELAPIDAE are generally more toxic than the venoms of VIPERIDAE as is evident from Table I, where the toxicities of venoms of some snakes belonging to ELAPIDAE, VIPERIDAE and CROTALIDAE which are generally responsible for causing most of the deaths, are recorded (1). The toxicity of a venom depends upon many factors, such as seasonal changes, lenght (size) of the snake, amount of venom collected, freqnency of milking, whether or not found in the field (forest) or taken from snakes in captivity, etc. The toxicity of a venom also varies with the nature of the animal employed for the experiment (2).

Venom is a mixture of several proteins. Relatively few of them have been separated until recently. Partial or complete separation of them has been achieved by employing such procedures as Am_2SO_4 fractionation, starch-gel electrophoresis, paper electrophoresis and anion and cation exchange chromatography. Nearly all venoms depending upon the species of origin contain at least eight to ten (sometimes more) well characterized components. Most of them if not all, are enzymes; each one is different from the other in their behaviour towards substrates. Their activities vary from species to species and even from venom to venom, collected from the same snake, at different seasons of the year (3).

The enzymes that are commonly found in the venom of all species belonging to ELAPIDAE, CROTALIDAE and VIPERIDAE, are phospholipase A, L-amino acid oxidase, deoxyribonuclease, ribonuclease, phosphodiesterase, non-specific phosphomonoesterase, 5'-nucleotidase, adenosine-5'-triphosphatase and are recorded in Table II. Proteases and amino acid esterases are generally found in the venoms of VIPERIDAE and CROTALIDAE. Cobra venom exhibits only feeble proteolytic activity, but contains several peptidases. Venoms of ELAPIDAE in contrast to those of VIPERIDAE contain enzymes capable of hydrolyzing acetylcholine and non-cholinesters (4). The venom of Formosan cobra contains besides acetylcholinesterase, an inhibitor of this enzyme too, which can be reversibly blocked by

TABLE I — RELATIVE TOXICITIES OF VENOMS OF SOME COMMON SNAKES OF ELAPIDAE, VIPERIDAE AND CROTALIDAE

SNAKES	Estimated MLD for Man (150 lbs. body weight) in mgs.
ELAPIDAE	
Bungarus candidus (blue krait)	1.5
Notechis scutatus (tiger snake)	2.0
Naja naja (Indian cobra)	20.0
Dendroaspis angusticeps (mamba)	20.0
CROTALIDAE	
Bothrops atrox (fer-de-lance)	70.0
Crotalus atrox (Western diamondback rattlesnake)	140.0
Crotalus adamanteus (Eastern diamondback rattlesnake)	280.0
VIPERIDAE	
Vipera russellii (Indian daboia)	50
Bitis arietans (African puff adder)	120

TABLE II

Enzymes	Cobra venom	*Vipera russellii* Venom	*C. adamanteus* Venom	*C. atrox* Venom
5'-Nucleotidase (AMPase)	+	+	+	+
Diphosphopyridine nucleotidase (DPNase)	+	not known	+	+
Adenosine triphosphatase (ATPase)	+	+	+	+
Deoxyribonuclease (DNase)	+	+	+	+
Ribonuclease (RNase)	+	+	+	+
Phosphodiesterase	+	+	+	+
Phosphomonoesterases (nonspecific)	+	+	+	—
Phospholipase A (in some venoms in addition to this Phospholipase B or C is found)	+	+	+	+
Protease	+	+	+	+
L-amino acid oxidase	+	+	+	+
Acetylcholinesterase (and cholinesterase)	+	—	—	—
ESTEROLYTIC ENZYMES IN SNAKE VENOMS				
Proteases				
Proteolytic enzyme (protein degrading enzyme)	+		+	+
Synthetic amino acid esters	+		+	+
Clotting enzymes	—	+	+	—
Bradykinin — releasing enzyme	—	not known	+	+
Bradykinin — destroying enzyme	—	not known	+	+
Clotting inhibitor (inhibits clotting due to its action on phopholipids)	—	—	—	+

Mg ions (5). Cobra venom exhibits adenosine diphosphatase and pyrophosphatase activities in addition to its well known ATPase activity although the activities of these two enzymes are low (6). Russell's viper venom can initiate coagulation of blood, whereas cobra venom acts as an inhibitor. The presence of phospholipase B in venoms of *Pseudechis porphyriacus* (Australian snake), *C. adamanteus* and *A. piscivorus* (North American snakes) has been recently reported (7). Some venoms also exhibit phospholipase C activity.

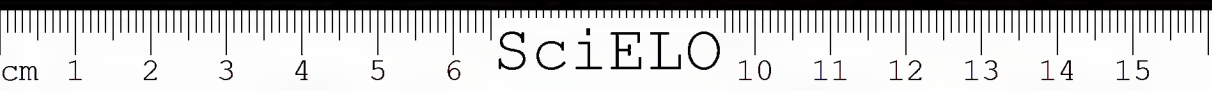
Although many of the enzymes found in the venoms of ELAPIDAE, VIPERIDAE and CROTALIDAE are common but the cause of death appears to be different in each case, e.g. it is generally believed that the primary cause of death in cobra poisoning is the failure of respiratory system brought about either by the paralysis of the central nervous system or by the paralysis of diaphragm muscle (8-11). The heart is affected at a much later stage. The changes in blood pressure following the intravenous injection of cobra venom into anesthetized animals, have been recorded by many workers; they all invariably found failures of respiration and circulation (8-13). Many of these investigators believed that the heart was primarily and directly affected (8, 9-13). Feldberg and Kellaway (14) pointed out that the circulatory failure was secondary to the effects of the venom on other organs. Marked electrographic changes in the rhythm and conduction of the heart resulting from an intravenous administration of *C. d. terrificus* venom led Soaje-Echagüe to consider myocardial inefficiency as the primary cause of the failure of circulation (13).

The action of cobra venom on isolated frog heart has also been extensively studied by many workers (11, 15-19). They all found an initial increase in the amplitude of contraction followed by complete stoppage of all the movements of an isolated frog heart, when it was perfused with venom solution. The effect as noted by most of the workers depended largely on the concentration of the venom solution used.

These studies, carried out *in situ, in vivo* and *in vitro,* indicate that the observed effects of the venom may be explained on the basis of its action on myocardium. Whether or not it was actually so, was investigated many years ago in our laboratory (19). The work to be presented here was based on three different series of experiments and was initiated to determine the sequences and relationship between the changes that follow after the intravenous injection of venom into animals.

Methods and materials

The experimental procedures employed in this study can be briefly described as follows: For the study of the effect of the venom on circulation and respiration, adult cats weighing between 2.0 to 2.5 kg. were chosen. Urethane (1.8 g/kg. of body weight) was introduced intramuscularly into the animal two hours prior to dissection. Femoral vein was cannulated for the administration of the venom (neurotoxin or cardiotoxin) dissolved in 2 ml. of saline. Carotid artery was cannulated for measurement of the changes of blood pressure, and the changes in respiration where recorded by means of a pneumograph.

For the study of the effects of cobra venom on excised heart, toads of average weight of 100 to 120 gms. were chosen. The heart was perfused with solutions of cobra venom (neurotoxin or cardiotoxin) of desired concentration. The contractions of the heart where recorded on a kymograph. The heart was

first perfused with the Ringer solution until it became physiologically normal; at this point. the cannula was filled with 2 ml. of the Ringer-venom solution of desired concentration and the drip started and the concentrations recorded. When all the venom solution was used up, it was replaced by Ringer solution.

The study of the effects of the venom (neurotoxin or cardiotoxin) on the transmission of nerve impulse were carried out with frog nerve-muscle preparations kept immersed in venom solution for different lenghts of time. The contraction of the muscle was elicited by indirect (through the nerve) and direct electrical stimulation.

The venoms were collected from snakes of the same species (*Naja naja*) and the same size, as far as possible, during the months of April and June, freeze-dried, and kept in an ice box (2-4°C) in a dark-brown stoppered glass bottle until use.

RESULTS

The results obtained can be summarized as follows: Following the injection of a large dose of cobra venom (2 mg/kg of body weight). a sharp fall in systemic arterial pressure is noted. The ensning respiration becomes more and more shallow and rapid, and tends to be abdominal in type until finally ceases; the B.P. drops to zero mm Hg and the animal dies, apparently because of the failures of respiration and circulation. The application of artificial respiration can not prevent the death of the animal. Even if a smaller dose of the venom (0.5 mg/kg of body weight) is administered, the changes in respiration and blood pressure follow the same patterns. If artificial respiration is applied, the heart gradually improves, B.P. slowly and progressively rises until it is restored to normal level. If the artificial respiration is discontinued at this point, natural respiration revives slowly and the animal survives for many hours. If now a larger quantity of venom (1.5 mg/kg) is administered, blood pressure drops almost instantaneously to zero and respiration ceases immediately. The lethal effects of this second dose can not be counteracted by the application of artificial respiration. If on the other hand, a smaller dose (0.2 mg/kg) of venom is initially introduced, a very slight fall in arterial blood pressure often accompanied with certain irregularities in respiration, is observed. If no further venom is administered, the B.P. slowly rises until it reaches the normal level and remains steady thereafter. Administration of three to four doses increasing each time the amount of venom introduced, (totalling 2.25 mg/kg) at regular intervals, does not affect either B.P. or respiration. At the end of the fourth administration, a large quantity of venom can be introduced even in a single dose, without affecting either the B.P. or respiration. After 4 to 6 hours, B.P. drops to zero, respiration ceases and the animal dies. If the heart is examined just immediately after the death of the animal, it is found in systolic contracture.

If neurotoxin, purified from the same venom, is injected into anesthetized cats, failures of respiration and circulation are observed almost at the same time. The latter can be restored to normal level if artificial respiration is applied, which remains steady thereafter for many hours (6 to 8 hours) until the animal dies because of other secondary effects. If the chest is opened and the heart is examined, no systolic arrest of the heart like the one that has been noted before with crude venom can be detected (19).

If the venom is heated through various temperatures to destroy one or the other active constituents of the venom and tested thereafter for its action on

respiration and circulation, it becomes evident that it retains this property until it is heated to 85°C for 30 minutes. At this temperature most of the enzymes are destroyed. Hemolysin which is not destroyed at this temperature is ineffective in causing respiratory and circulatory failures.

Recently Vick and his colleagues isolated from cobra venom three major physiologically identifiable components (actually 12 components were separated). The first component, which was a mixture of 3 to 4 separate fractions produced a loss of cortical electrical activity when injected intravenously into dogs. The second component (fractions 5 to 8) causes respiratory paralysis and the third component (fraction 12) affected cardiovascular system. On the basis of the effect of fraction 12 on arterial blood pressure and heart rate, these workers concluded that cobra venom has a definite action on cardiovascular system of the dog.

Action of cobra venom on excised frog heart

If an isolated frog heart is perfused with an extremely diluted solution of venom (1 in 100,000) no changes in the amplitude of contraction could be noticed; no stimulating or depressing action of venom could either be detected if the heart is perfused with a less diluted venom solution (1 in 10,000). An increase in the amplitude of contraction for a brief period is noticed when a venom solution of 1 in 5,000 is used, which disappears on washings and the heart becomes normal. If a more concentrated solution is used, say 1 in 1,000, an increase in amplitude is first observed and then certain irregularities and slowing of heart beats are noticed. The heart finally stops beating. These effects of the venom can also be partially removed by prolonged washings with the Ringer. If a more concentrated venom solution (1 in 200) is used, no stimulating action can be detected, instead, a progressive decrease in the amplitude of contraction is observed. The heart finally stops in systolic contracture and can not be revived in this case even after continuous washings with Ringer. The effects of venom on excised heart could not be counteracted by atropine and antihistamine drugs.

If the heart is perfused with a venom solution (1 in 200) previously heated through various temperatures, it is found that venom loses its action at 85°C (20). Phospholipase A or neurotoxin has no such effect on excised heart.

It is interesting to note that similar findings have been recorded with different concentrations of digitalis drugs (digitalis, strophantheine, etc.). In all cases where high concentrations of these drugs have been used, the heart stops in systolic contracture but contrary to the action of the venom, their effects could be easily washed out with Ringer. Recent histopathological studies indicate that venom produces specific changes in the isolated hearts of frogs and of guinea-pigs which are quite different from those produced by histamine, strophantheine, etc.

When an isolated frog-nerve preparation is exposed to a venom solution, it loses its excitability in 4 to 6 minutes depending upon the concentration of the venom solution used (21, 22). Neither direct nor indirect stimulation can produce contraction. The ability of the electrical stimulus to elicited contraction when applied through the nerve is lost earlier than if applied directly to the muscle. The muscle contracts to one third of its original lenght, becomes pale and opaque. Nerves or muscles exposed to only Ringer's solution maintain electrical property

for 4 to 6 hours. If the nerve-muscle preparation is exposed to neurotoxin, the muscle retains its excitability; under identical conditions only muscle loses its excitability if exposed to cardiotoxin solution. The ability of venom to block contraction of the muscle is lost if it is heated at 55°C for 10 min. or 85°C for 30 min., depending upon how the contraction is elicited. i.e. whether stimulated through the nerve or through the muscle (21). At these temperatures acetylcholinesterase activity of the venom and its cardiotoxic property are destroyed. The action of cardiotoxin can not be prevented or counteracted by K ions, antihistamine drugs or by EDTA, known to inhibit the protease action of the venom. Cobra venom or cardiotoxin thus appears to have direct paralysing action on cardiac and skeletal muscle but how it acts is not known. Neither cobra venom nor cardiotoxin can produce any contraction of actomyosin fibers either extracted directly from rabbit skeletal muscle or prepared synthetically by mixing crystallin myosin and actin. It has neither any action either on myosin or actin (23). It thus appears that venom has no action on individual components that constitute actomyosin. the contractile element of the muscle. Its action is visible when only intact muscle cells are involved.

The results obtained indicate that the observed changes in blood pressure that occurred following the injection of cobra venom, might be due to its direct action on myocardium rather than on central nervous system since neurotoxin has no such effect. It is possible that cobra venom contains another factor responsible for all the observed changes in the circulation when the venom is injected into an anesthetized cat and the changes in the movements of an isolated frog heart when it is perfused with the venom solution. This factor was isolated from the venom using the conventional methods which involved heat denaturation, fractional precipitation with Am_2SO_4, adsorption and elution from Ca-phosphate-gel, iso-electric precipitation, etc. (24). The purified material was 20 times more active than the crude venom, and the molecular weight as determined by diffusion method was found to be 30,000 (25). Recently it has been purified using such methods involving heat denaturation, Am_2SO_4 fractionation, chromatography on DEAE-column and starch-gel electrophoresis. The molecular weight of this purified material as determined from sedimentation constant, is approximately 13,000 to 14,000. It is 30-35 times more active than crude venom.

Curiously enough, when a single dose of cardiotoxin (0.1 mg/kg) is administered intravenously into an anesthetized cat. an immediate sharp fall in arterial blood pressure to zero mm Hg is observed; respiratory failure also occurs at the same time. Artificial respiration if applied fails to restore the blood pressure to normal level. When the heart is examined, it is found in systolic contracture. If an isolated toad heart is perfused with a cardiotoxin solution, there is at first some augmentation in the amplitude of the contraction for a brief period followed by a gradual decrease in amplitude until finally all the movements are stopped. The heart goes to systolic contracture. The effect of cardiotoxin can not be washed out by prolonged washing with Ringer. It has no action on neuromuscular junction nor on nerves but skeletal muscle kept immersed in cardiotoxin solution loses its excitability.

The results so far obtained, indicate that all the effects recorded either with crude cobra venom or cardiotoxin suggest that these effects are not possibly due to the action of histamine or histamine like compounds, released from tissues by the action of cobra venom or cardiotoxin, as the latter has neither any proteolytic activity nor any phospholipase A activity and can not be implicated in

releasing histamine or histamine like compounds from mast cells. There are many reports in the literature supporting the hypothesis that the effects of venom are due to the action of histamine liberated. They are mainly based on reports of successful treatment of snake bites with antihistamine drugs, ACTH (adreno-corticotropic hormone) and cortisone (26-28). These reports are no doubt of some interest to us but are not as significant as imagined since a critical analysis of the results reveals many short comings which do not allow to draw any statistically justified conclusions as to either therapeutic values. This is due to the lack of reliable standard by which the severity of snake bite poisoning can be judged and because of the limited number of cases treated successfully. The merits of these drugs have been therefore reassayed in our laboratory by more accurately controlled animal experiments.

The antihistamine drug used was 10-(20-dimethylaminoisopropyl)-phenothiazine. The cortisone was a commercial preparation of 11-dehydro-17-hydroxy-corticosterone-21-acetate obtained in the form of a suspension in saline. The doses of ACTH or cortisone administered were 25 IU per kg of body weight. The drugs were administered in all cases along with the venom.

The results obtained with these drugs clearly indicate their ineffectiveness to counteract the action of cobra venom on circulation and respiration; in other words, they can not prevent the fall of arterial blood pressure to zero mm Hg and counteract the respiratory failure that occur following the administration of the mixture. None of the drugs can also prevent the stoppage of the movements of the excised frog heart when it is perfused with the venom or cardiotoxin solution, they can not either counteract the action of cobra venom on neuro-muscular junction or muscle fibers. Schöttler (29) did not also find any beneficial effect of antihistamine drugs in snake poisoning in contrast to its appreciable curative effect against fatal dose of histamine. He did not also find any effectiveness of ACTH, cortisone, etc., against venom poisoning.

Cardiostimulating principle in cobra venom

If an isolated (detached from the body) frog heart is kept immersed in a venom solution, it loses its ability to produce rhythmic movements in 6 to 10 minutes depending upon the concentration of the venom solution used. If it is exposed to a very diluted solution say 1 in 5,000, it takes 2 to 3 hours to stop heart beat. If on the other hand the heart is exposed to a venom solution previously heated at 98°C for one hour, the heart continues to beat for as long as 12 to 14 hours. The rhythmic movements become more and more feeble with time. Under identical conditions, the heart continues to beat for only 4 to 6 hours if kept immersed in Ringer solution.

Since at 98°C all the known components of the venom with exception of neurotoxin are destroyed and the purified neurotoxin has no such effect on heart or skeletal muscle, it may be possible that the factor directly responsible for maintaining the rhythmic movements of the heart for 12 to 14 hours, has been masked by the presence of other factors, particularly by cardiotoxin, which has just the opposite effect on heart. This factor may be associated with cardiotoxin; and without the destruction of the latter, the stimulating effect of venom could not be detected. If instead of the heated venom solution the isolated heart is exposed to a mixture of triphosphates of all the four nucleosides, ATP, GTP, CPT and UTP or TTP in equivalent proportions, the heart continues to beat

for only 6 to 8 hours. When such a mixture is replaced by any individual nucleotide say AMP, GMP, CMP, UMP or TMP only a slight stimulating effect is observed with AMP.

Since the isolated heart continues to beat for 12 to 14 hours in heated venom solution and the fact that none of the four nucleotides either individually or collectively can maintain the rhythmic movements of the heart longer than 6 to 8 hours, it is believed that the factor responsible for the stimulating effect of the venom is a protein, quite resistant to heat and has low molecular weight. Venom loses this property if it is heated at 100°C for one hour. This factor is a cardiostimulating factor, as opposed to cardiotoxic factor. Its separation and characterization are now under active investigation in our laboratory.

Cobra venom contains besides neurotoxin and enzymes, two more factors. The one, cardiotoxin, is responsible for the failure of circulation and stoppage of all the movements of an excised heart. These effects can not be abolished or reversed by any prolonged washing or by any antihistamine drugs, atropine or corticosteroids. Since the effect of venom or cardiotoxin is primarily due to either action on cardiac or skeletal muscle rather than on central nervous system, it is believed cardiotoxin should be the most appropriate name that can be assigned to this factor. The other factor stimulates the heart, its effect is only visible when the other factor is destroyed.

The present communication emphasizes the importance of the isolation of various components in relatively purer form, and the study of their nature, biochemical, and pharmacological actions separately as well as collectively. Such comprehensive studies are believed to be more useful and significant in the understanding of the cause of death from snake bites.

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60. CARDIOVASCULAR RESPONSES TO SNAKE VENOMS AND THEIR FRACTIONS

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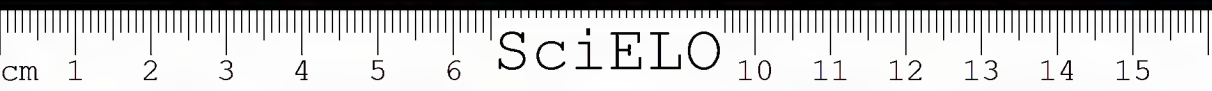
It has long been known that certain snake venoms exert a deleterious effect on the cardiovascular system. During more recent years, attempts have been made to determine which fraction or fractions of snake venoms are responsible for these deleterious effects. Some attempt has also been made to define the relationships between the direct effects of the venom on the cardiovascular system and those which may be precipitated by autopharmacological changes initially provoked by the venom. In the course of these various investigations, many important data, which indicated the complexity of the vascular response, have been obtained. The purpose of the present paper is to present a short review of the compendium of knowledge on the cardiovascular effects produced by snake venoms, and to reflect on some experiences which, I feel, are important as guide lines for future research on the pharmacology of these fascinating toxins.

First, I should like to discuss, in a very general way, some relationships between the physiopharmacological activities of snake venoms and their chemistry. We are well aware, as has been pointed out by numerous workers, that snake venoms are complex mixtures, chiefly proteins, many of which have enzymatic activities. There was a time in our thinking when *all* of the deleterious effects of venoms were linked with the enzymatic components of the toxin. We now know, of course, that this is not true, and indeed, the more lethal activities of most venoms — whether they be snake, arthropods or fish — are not generally associated with one enzyme or even with several, although it does appear that certain enzymes may be closely bound with certain non-enzymatic proteins in a venom, and that these combinations can be quite lethal. On the other hand, the enzymes of snake venoms are certainly responsible for some of the deleterious changes provoked by the crude toxin, and perhaps in particular with those changes which appear to occur at the cell membrane, whether the cell be one in the intima of a vessel or in subcutaneous tissues, or even if it be a blood cell. These unusual combinations of multiple enzymatic and non-enzymatic proteins, along with certain non-protein substances, not only indicate very complex organization and physiopharmacological activity, but might appear to reflect upon a series of adaptive mechanisms during the evolution of the venom. On the other hand, snake venom enzymes may be involved in far more complex biological functions, as has long been proposed by Professor Zeller in his many fine papers. I am sure we are all becoming impressed with the fact that, in general, there is a great deal of conformity in the organization of the chemistry of snake venoms, and while certain fractions are indeed quite different, chemically as

well as physiopharmacologically, particularly at the family level, there appear to be more similarities than differences than what we had thought one or two decades ago. This same general impression is also obtained when one considers the marine venoms and poisons, and indeed to some extent even with arthropod toxins.

There is a tendency, perhaps fortunately so, in the well-oiled scientific mind, to gather data into orderly groups for purposes of classification. This is an admirable trait but it can be fraught with some dangers, and perhaps nowhere in biology is this more evident than in our own field. In the absence of reliable data there has been a tendency to arrange snake venoms into loose indefinable classifications. These classifications have, in many instances, been more confusing than helpful. Even such widely used terms as *neurotoxin* and *cardiotoxin* are inadequate and misleading, for they are often applied to the whole venom, and most venoms are complex mixtures having several or many biological properties. It has also been shown that *neurotoxins* can, and often do, have *cardiotoxic* or *hemotoxic* activity, or both; *cardiotoxins* may have *neurotoxic* or *hemotoxic* activity, or both, and *hemotoxins* may have the other activities. The labeling of a venom as a *neurotoxin* is not only confusing but dangerous, for it may lead a physician to make unwise clinical decisions (1).

No one has brought this point closer to focus than H. Alistair Reid, who has recently demonstrated that the principal clinical feature of cobra poisoning in Malaya is local necrosis, and that *neurotoxic* effects in human victims are indeed rare (2). Furthermore, as Dr. Reid points out, and as has been noted by previous workers, the cardiovascular effects of these venoms are often times marked, and in many cases are the cause of death. It would seem, that until the fractions responsible for the deleterious effects of a snake venom have been isolated and studied individually and in combination, we need to exercise extreme care in systematizing data which are based partly on biological assay methods, partly on biochemical studies, partly on clinical observations and partly on intuitive hunches.

There is another weakness in our over-all approach to the study of the physiopharmacological properties of snake venoms. There is a tendency to link specific chemical structures with specific biological activities; i.e., that substance A produces effect A, and that substance B produces effect B, etc. It would appear to me that it would be highly unlikely and unbecoming for nature to have developed venoms in this manner. Particularly, since perhaps one of the most important factors in the evolution of a venom is the role played by the adaption of the prey or the offending animal. But whether or not this concern is founded, the very principle of looking for a specific fraction to exert a specific effect is a dangerous one, for among other things, it may often limit our concept and our experimental approach to determining the mechanism of action of a venom by thinking that it necessarily follows that *one* specific fraction has *one* specific function in or on *one* organ system, although in some cases this may prove to be true.

Some of the so called *neurotoxins* that have been sent to us for further evaluation in cardiovascular preparations during the past five years have had a more marked effect on the cardiovascular system than on the nervous system, and in some cases, in fact, this effect has far out-shadowed that on the latter system. In most of these cases the error in judgement has not weighed with the venom but rather with the experimenter, who, for example, having seen a mouse in convulsions following the injection of the venom has presumed that the

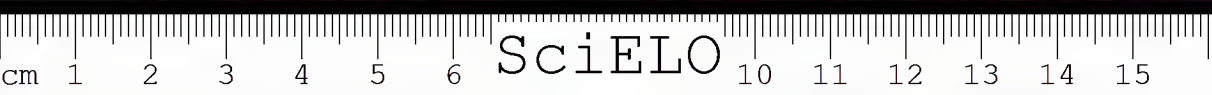
venom was a *neurotoxin*, when in reality the mouse is convulsing because of cerebral anoxia caused by a markedly reduced blood supply to its brain secondary to systemic hypotension.

There is one other area of cardiovascular research on which I should like to comment. This involves the choice of the proper experimental animal. One must be exceedingly careful in applying data derived from studies in one group of animals to conclusions about the biological effects of a venom in another group of animals or to data on the design, use and adaption of a venom. We are all familiar with the marked differences in the lethality of venoms for different animals and how in some cases these are particularly related to specific ecological problems. Unfortunately, much of our information on the zootoxicological properties of venoms is based on studies with mammals, which, of course, limits their application as far as our understanding the design of the toxin in the animal's armament. On the other hand, it is equally dangerous to apply data obtained on a fish nerve-muscle preparation or the frog heart, or the cockroach heart, to conclusions about the action of the venom on man, or even on a mammal. Certainly the more diversified our studies the more important data that we will obtain, but the application of these data must be guarded zealously.

Even in applying data obtained in mammals one must be exceedingly careful. For instance, data obtained from cardiovascular studies in dogs cannot be liberally applied to humans. It has become increasingly apparent, particularly from studies on shock, that the dog may respond quite differently than the human under similar experimental conditions. The importance of the portal circulation in the dog during various hypotensive crises is far more marked than in the human. In certain cardiovascular conditions the dog must be considered a *portal* animal while man certainly is not. Another difference is in the renal circulation. In the rabbit and cat, as well as in the human, the renal vein is known to dilate quite readily in response to mechanical and certain chemical stimuli. It is far less responsive in the dog, while on the other hand the splenic vein in the latter animal dilates quite easily with specific drugs and stimuli. This probably reflects the high degree of spleen reservoir function in this species. In the cat, rabbit and human the splenic reservoir is much less marked. In certain mammals, particularly the dog, certain venous values play a more important role in the vascular response than they do in the human. The cat, on the other hand, appears to respond to snake venoms in a manner much more like the human than does the dog. In both the cat and in the human the hypotensive crisis evoked by *Crotalus* venom, for instance, is associated with changes in the pulmonary circulation and perhaps in the larger vessels of the chest (1, 3, 4).

These few examples point out the great care one must take in applying data from one group of animals to another, or to humans. One might conclude that perhaps many of the differences noted in the literature between responses to various venoms are more directly related to the choice of animal used than the venom used or even to the technique applied.

Rather than deal with specific data on the cardiovascular effects of snake venoms and their fraction (and of these effects most of us are acquainted), I should like to review some of the basic concepts that one must consider if he chooses to measure and interpret the changes provoked by venoms in the dependent variables of the various parameters of the cardiovascular system.

Fig. 1 illustrates the principle of parallel circuits and resistances in the cardiovascular system. Since the cardiovascular system accomplishes its biological

functions in a mechanical way, it can be seen that any change in one of the parameters will affect changes in the resistance of one, several or all other parameters of the system. These changes will be reflected in the dependent variables, that is in the heart output, and the pressures on the arterial and venous sides of the systemic and pulmonary circulations. The relationships can be plotted as

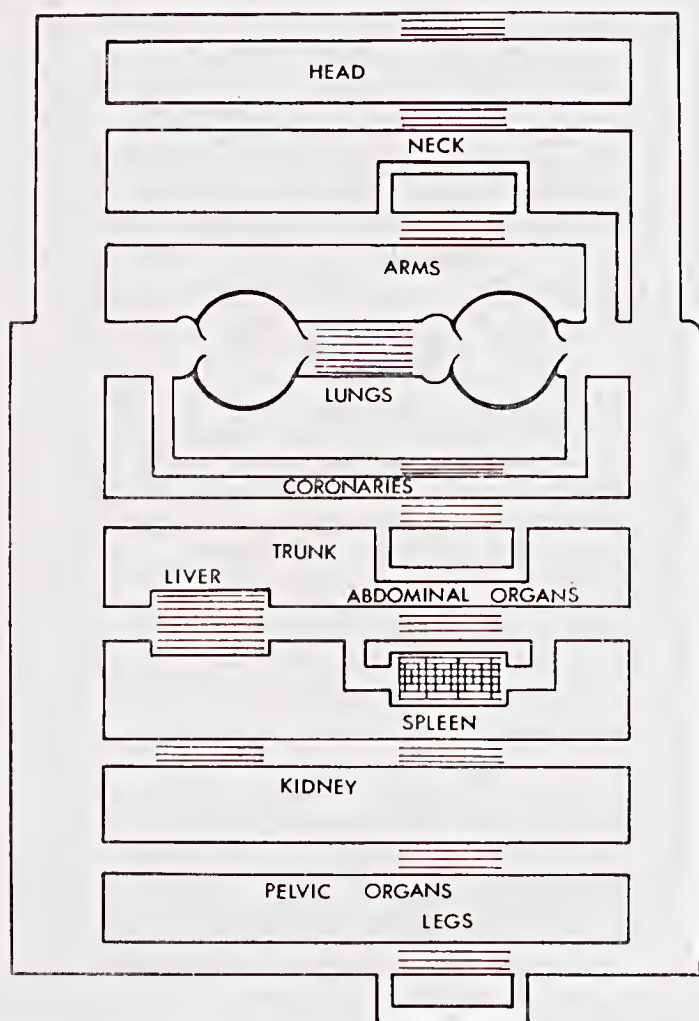



Fig. 1 — Diagram of the circulation showing parameters, including resistances (modified from Wezler and Böger, 1939).

a mathematical formulation, and this has been done by several workers. In 1954, before the days of electromagnetic flowmeters and other flow-pressure monitoring devices, Prof. Van Harreveld and I proposed a model of the circulation (Fig. 2), from which certain equations could be developed and used in defining more carefully the changes provoked in the vascular parameters by various venoms (5).

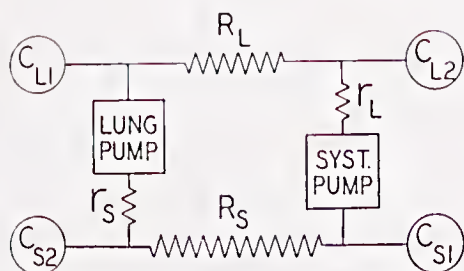



Fig. 2 — Circulatory model.

The relevant equations describing the relations between the parameters and the dependent variables of the circulation may be summarized as follows:

$$I = \frac{V}{C_{S1}(R_S + r_S) + C_{S2}\, r_S + C_{L1}(R_L + r_L) + C_{L2}\, r_L}$$

$$P_{S1} = \frac{V(R_S + r_S)}{C_{S1}(R_S + r_S) + C_{S2}\, r_S + C_{L1}(R_L + r_L) + C_{L2}\, r_L}$$

$$P_{L2} = \frac{V\, r_L}{C_{S1}(R_S + r_S) + C_{S2}\, r_S + C_{L1}(R_L + r_L) + C_{L2}\, r_L}$$

In these equations, I is the heart output. P_{S_1} represents the systemic arterial pressure. P_{L_2} indicates the left atrial pressure. V is related, though not synonymous, with the total volume of the circulating blood. R_S and R_L are the resistances in the arterioles and capillaries of the systemic and pulmonary circulations respectively (peripheral resistance). The total resistance in the venous system and all the factors limiting the blood flow into the ventricles are represented by the resistances r_S for the right heart and r_L for the left heart. The largest factor for the value r is the elastical resistance of the ventricular wall against filling. C_{S_1}, C_{S_2}, etc...., are the constants of capacitance (volume change per unit change of pressure) for the various components of the circulatory apparatus. These components are an index of the tone of the larger vessels of the systemic and pulmonary arterial and venous systems.

It can be seen that by varying a parameter one can determine, through measurements in the dependent variables, the pressures and flows in the various components of the cardiovascular system. Today, this has been made more simple by the advent of the electromagnetic flowmeter. It is now possible, with a little care, to measure blood pressure and flow concomitantly in a single vessel, and to carry out this procedure in four or five vessels during a single experiment. The information obtained from such measurements not only gives us valuable data on the changes themselves and their relationships to other vascular phenomena, but it gives us a ready insight into the mechanism of action of the venom.

This might be demonstrated by several recent experiments with *Crotalus* venoms. Using transducers to measure certain arterial and venous pressures on both sides of the systemic and pulmonary circulations (some measurements being

taken through catheters threaded through the heart under fluoroscopy), and by taking simultaneous blood flow records with electromagnetic flowmeters from the same and different vessels, and by concomitant recording of the electrocardiogram and electroencephalogram, and by measuring cerebrospinal fluid pressure and the rate and depth of respirations it has been possible to determine the sequence of events of the immediate and precipitous hypotensive crisis that occurs following the intravenous injection of *Crotalus* venom (3, 4). It has also been possible, using these techniques and certain isolated organ techniques (3, 4), to obtain some insight into the mechanism of action of this venom.

From these studies it has been concluded that the hypotensive crisis is due to changes in the resistance within the pulmonary circuit. These changes lead to a decreased left heart output pressure and flow, which in turn provoke alterations in the flow and pressure within various vessels, changes in peripheral resistance, changes in respiration, cerebrospinal fluid pressure, and in the electrocardiogram and electroencephalogram. It was suggested that the change in pulmonary resistance may be attributed to the pooling of blood in the lungs and larger vessels of the chest, due to post-capillary resistance from either vascular constriction and/or the formation of multiple thrombi. It appears that each of these phenomena may play a part in the crisis. These changes are evident in the human, monkey and cat. They are much less conspicuous in the dog, where the mechanism for the hypotensive crisis may be quite different.

The studies previously noted might serve as a guide for future work on the cardiovascular effects of venoms and venom fractions, for the techniques provide a considerable amount of data not otherwise obtainable, while also permitting a careful check and evaluation of interpretations. But even today new techniques are being developed, and these may give us considerably more insight into the mode of action of toxins than we anticipate. Particularly encouraging is the possibility of measuring definitive vascular changes with tagged venom and blood, although even these tools have obvious limits of usefulness. In all of these studies, however, the critical contribution will still rest with the experimenter. As one studies the progress in our science, as in all sciences, it is obvious that the most significant contributions are fully dependent upon the investigator and not upon his equipment. The cardiovascular effects of venoms and their fractions are slowly being unraveled by scholars and not by gadgets, and we have seen in this meeting a demonstration of the kind of intellectual stimulation and encouragement that will greatly enhance our understanding of the properties of these most remarkable toxins.

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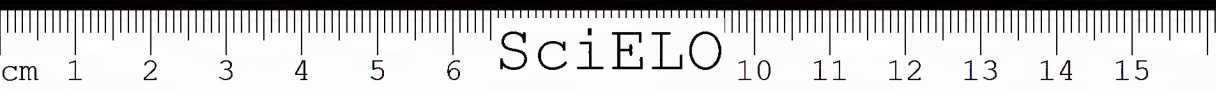
61. PHARMACOLOGY OF THE COMPONENTS OF TOAD VENOM AND ALLIED SUBSTANCES

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The dried venom of the Chinese toad has been used externally from antiquity as a home remedy called Senso (Ch'an Su) for canker sores, tooth-ache, and many local inflammatory conditions. But most pharmacologists have concentrated themselves on the study of its cardiotonic action, and little is known on its local actions.

We have investigated the local anesthetic action of each purified substance extracted from Senso (Table I). As described in previous papers (1, 2, 3) the strong local anesthetic property was found generally in the steroid fractions while the water-soluble fractions have local irritating action with very weak or almost no local anesthetic properties. For example bufotenidine, a water-soluble derivative of tryptamine, has no anesthetic action.

For pharmacological tests, the substances insoluble in water were dissolved in a mixture of water and propylene glycol. In those cases control tests were made with the same concentration of propylene glycol.

Surface anesthetic action on cornea was determined with adult male rabbits. The conjunctival sac was filled with 0.2 ml of the solution of various concentrations, and the lids were released after one minute. The wink reflex was elicited by a horse-tail hair (0.17 mm in diameter, 0.7-0.75 g in pressure). The test of 6 pricks was applied on cornea every 5 minutes for 30 minutes. The number of times the prick failed to elicit a blink reflex during the 30-minute period was added up and the sum gives an indication of the degree of anesthesia. Therefore, $6 \times 6 = 36$ is the value that indicates complete anesthesia for the 30-minute period. Tables II and III show some results.

When these results for each substance were plotted on a log dose-response coordinate, the approximately straight lines so obtained were not quite parallel to each other, which makes it difficult to state exactly the relative strength of anesthetics to cocaine (Fig. 1), but in round figures, when anesthetic potency of cocaine hydrochloride is taken as 1, that of procaine hydrochloride is 0.1, while that of Fraction No. 200 is 30-60 (mean 40) as Table IV shows. This fact is remarkable and what is more, No. 200 has almost no irritating action.

TABLE I

Senso (dried toad venom) (powder)
(No. 192)
Petroleum ether
soluble part
insoluble part
extracted with ether
for 2 months
(40-45°)
insol. p.
sol. p.
(No. 193)
ethan
extracted 7 times
with methanol
(60°C)
insol. p.
(No. 195)
sol. p.
(No. 194)
insol. p.
sol. p.
(No. 196)
concentrated in low pressure of CO2
under 60°, added to water slightly
acidified with HCl, and stirred
aq. sol. p.
(No. 197)
Bufotenidine
(No. 190)
water-insol. p.
(No. 198)
CHCl3
insol. p.
sol. p.
stirred with 1% HCl and shaken with
CHCl3
CHCl3-soln.
HCl-soln.
stirred with 5%
NaHCO3
CHCl3-soln.
NaHCO3-soln.
washed with water
CHCl3-soln.
aq. soln.
Al2O3-column chromatography
partitioned
(No. 199)
(No. 200)
(No. 201)

TABLE II — NUMBER OF TIMES THE BLINK REFLEX IS ABSENT DURING A 30-MINUTE PERIOD (No. 200)

Time (min)	CONCENTRATION															
	0.011%								0.017%							
5	4	2	3	2	2	2	3	2	5	4	5	5	4	2	3	4
10	5	5	6	5	4	5	5	5	6	6	6	6	6	6	6	6
15	4	2	4	5	1	3	4	5	6	6	6	6	6	6	6	6
20	2	0	2	3	0	0	2	3	6	4	6	6	5	6	5	2
25	0	0	0	1	0	0	0	0	4	3	4	4	4	4	4	0
30	0	0	0	0	0	0	0	0	1	1	2	2	2	3	2	0
Total	15	9	15	16	7	10	14	15	28	24	29	29	26	27	26	18

Time (min)	0.025%							
5	5	4	6	6	5	4	5	6
10	6	6	6	6	6	6	6	6
15	6	6	6	6	6	6	6	6
20	6	6	6	6	6	6	6	6
25	6	5	6	6	6	6	5	6
30	3	3	6	6	5	6	5	6
Total	32	30	36	36	34	34	33	36

TABLE III — NUMBER OF TIMES THE BLINK REFLEX IS ABSENT DURING A 30-MINUTE PERIOD. COCAINE-HCl

Time (min)	CONCENTRATION															
	0.2%								0.4%							
5	4	6	6	6	6	6	6	5	6	5	6	6	6	6	6	6
10	1	3	6	3	3	3	2	2	6	6	6	6	6	6	6	6
15	0	0	2	0	0	1	0	0	2	3	6	6	5	4	4	4
20	0	0	0	0	0	0	0	0	0	0	1	4	0	2	1	0
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	5	9	14	9	9	10	8	7	14	15	19	22	17	18	17	16

Time (min)	CONCENTRATION															
	0.8%								1.6%							
5	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
10	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
15	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
20	2	4	6	5	6	5	5	5	6	6	6	6	6	6	6	6
25	0	0	3	2	2	0	3	2	6	4	6	6	6	5	6	6
30	0	0	0	0	0	0	0	0	6	0	6	5	4	2	5	6
Total	20	22	27	25	26	23	26	25	36	28	36	35	34	31	35	36

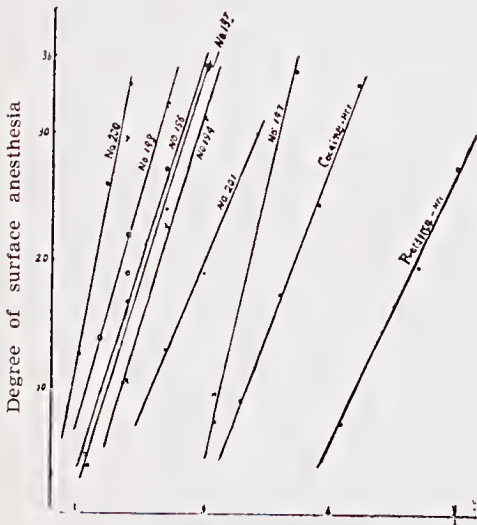



Fig. 1 — Log Dose-Response curves.

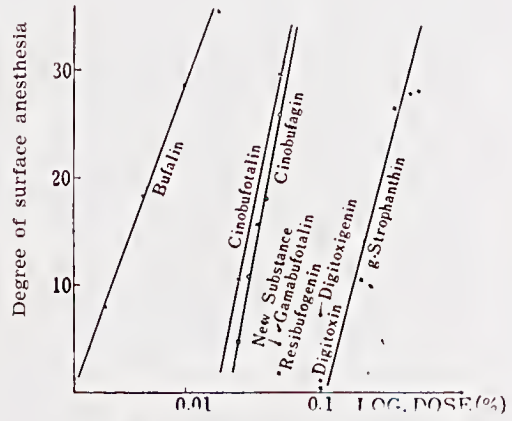



Fig. 1a — Log Dose-Response curves of Bufogenins and allied Compounds.

TABLE IV — POTENCY IN TERMS OF COCAINE-HCl (=1.0). SURFACE ANESTHESIA IN RABBIT CORNEA

Substance	Number of animals	Potency of surface anesthesia			
		R_1	R_2	R_3	Mean
Cocaine-HCl	8	1	1	1	1
Procaine-HCl	6	0.13	0.11	0.09	0.11
193	6	12.6	14.8	17.4	14.9
196	6	13.8	16.2	19.1	16.4
194	6	9.6	11.2	13.5	11.4
197	6	1.6	2.2	3.2	2.4
198	6	19.1	24.6	28.8	24.2
199	4	0	0	0	0
200	8	28.8	39.8	57.5	42.1
201	6	5.0	4.7		

Antilog (X_c-X_s) X_c: log dose of cocaine-HCl

X_s: log dose of substance

R_1: Response 10-level

R_2: Response 20-level

R_3: Response 30-level

The onset of local anesthesia by No. 200 is somewhat slower than cocaine, but its duration is much longer. In infiltration anesthesia test (wheal method) on man, this substance (No. 200) produces total local anesthesia in 5 minutes, which continues during 30 minutes to 1 hour in 0.0003% solution and for 3 hours in 0.03% solution, and no local tissue damage was left.

The fraction No. 200 corresponds to the substance of fraction number Fr. 52 in the report by Ohno and Komatsu (4). As was therein described, this substance gave four spots with different Rf values by paper partition chromatography, but repeated purification finally afforded a substance giving only a single spot at Rf 0.61.

Komatsu (3) examined the chemical and physical properties (melting point, elementary analysis, Rf value, colour reaction, ultraviolet and infrared spectra) of this fraction and found it to be identical with bufalin isolated from toad venom by Kuno Meyer. Local anesthetic activities of Meyer's sample of bufalin (No. 208) and the sample (No. 209) isolated by Komatsu were compared and we found no difference between the two, both qualitatively and quantitatively.

The assay method for local anesthetic activity was the same as previously described, and the results are shown in Tables V and VI. Samples No. 208 and No. 209 in 0.0025% solution showed the maximum hypesthesia after about 10 minutes and the drug action disappeared after about 20 minutes. With 0.005% solution, hypesthesia continued for about 30 minutes, with 0.01% solution for over 30 minutes, and an almost perfect anesthesia was exhibited. With 0.02% solution, complete anesthesia continued for over 30 minutes and there seemed to be no irritation on the cornea.

TABLE V — NUMBER OF TIMES THE BLINK REFLEX IS ABSENT DURING A 30-MINUTE PERIOD — No. 208

Time (min)	CONCENTRATION											
	0.0025%						0.005%					
5	0	2	0	2	2	0	2	4	5	2	2	3
10	3	4	3	4	3	4	6	6	6	6	5	6
15	3	1	2	3	3	3	6	5	4	5	5	5
20	0	0	0	2	1	2	5	4	3	4	3	4
25	0	0	0	0	0	0	3	2	1	0	0	1
30	0	0	0	0	0	0	0	0	0	0	0	0
Total	6	7	5	11	9	9	22	21	19	17	15	19

Time (min)	CONCENTRATION											
	0.01%						0.02%					
5	5	5	5	4	5	3	6	6	6	6	6	6
10	6	6	6	6	6	6	6	6	6	6	6	6
15	6	6	6	6	6	6	6	6	6	6	6	6
20	6	6	6	6	6	5	6	6	6	6	6	6
25	5	4	5	6	4	3	6	6	6	6	6	6
30	4	2	3	3	1	1	6	6	5	6	6	6
Total	32	29	31	31	28	24	36	36	35	36	36	36

TABLE VI — NUMBER OF TIMES THE BLINK REFLEX IS ABSENT DURING A 30-MINUTE PERIOD — No. 209

Time (min)	CONCENTRATION											
	0.0025 %						0.005 %					
5	2	0	0	2	2	2	4	4	3	3	3	3
10	4	3	0	4	4	4	6	6	5	6	5	5
15	2	3	0	3	3	1	6	5	5	5	4	5
20	0	1	0	2	1	0	5	3	4	4	3	3
25	0	0	0	0	0	0	3	1	1	2	1	2
30	0	0	0	0	0	0	1	0	0	0	0	0
Total	8	7	0	11	10	7	25	19	18	20	16	18

Time (min)	CONCENTRATION											
	0.01 %						0.02 %					
5	5	5	5	5	4	4	6	6	6	6	6	6
10	6	6	6	6	6	6	6	6	6	6	6	6
15	6	6	6	6	6	6	6	6	6	6	6	6
20	5	6	5	6	4	5	6	6	6	6	6	6
25	4	5	5	5	3	4	6	6	6	6	6	6
30	1	3	3	4	1	2	6	6	6	6	4	6
Total	27	31	30	32	24	27	36	36	36	36	34	36

Dose-response curve is given in Fig. 2. Comparison of the values from this graph with those of cocaine hydrochloride and procaine hydrochloride is given in Table VII.

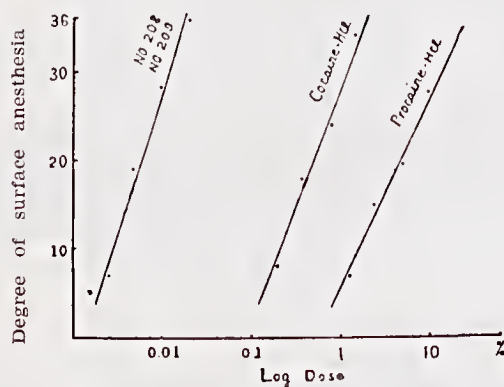



Fig. 2 — Log Dose-Response Curves.

The local anesthetic action of samples No. 208 and 209 is over twice that of No. 200, and about 90 times that of cocaine hydrochloride, and duration of their action is markedly longer, although it takes somewhat longer time for the action to appear.

TABLE VII — POTENCY IN TERMS OF COCAINE HYDROCHLORIDE (=1.0) SURFACE ANESTHESIA IN RABBIT CORNEA

Compound	No. of animals	Potency of surface anesthesia			
		R_1	R_2	R_3	Mean
Cocaine-HCl	8	1.0	1.0	1.0	1.0
Procaine-HCl	6	0.12	0.11	0.09	0.11
No. 209	6	78.7	87.9	98.0	88.2

Antilog (X_c-X_s) X_c: log dose of cocaine-HCl — R_1 Response 10-level
X_s: log-dose of substance — R_2 Response 20-level
R_3 Response 30-level

This fact was also confirmed in intradermal wheal test on human skin. In this test 0.1% solution (in 50% propylene glycol) of No. 209 was diluted with physiological saline to make 0.01% and 0.001% solutions, 0.1 ml of each solution was injected intracutaneously in the forearm, a local patch of anesthesia was produced and its degree and duration was tested by pin-pricks.

With 0.001% solution, anesthesia lasted for about 30 minutes, while with 0.01% solution complete anesthesia lasted for several (3-7) hours, without leaving any tissue damage. These results are in marked contrast with the effect of cocaine hydrochloride and procaine hydrochloride, whose intracutaneous injection of 0.1 ml of 0.125% solution causes immediate appearance of the action but its durations are respectively 15 and 6 minutes.

The local tissue-irritating properties of these fractions were estimated by intradermal injection of 0.3 ml of the following local anesthetics into the clipped abdominal skin of a rabbit by Trypan Blue test described by Hoppe, Alexander, and Miller (5) and the magnitude and intensity of local blue spots on the abdominal skin caused by the intravenous injection of trypan blue (1%, 1 ml/kg) were measured after 30 minutes, 1 hour, and 3 hours. With 2% or 5% procaine-hydrochloride (5% propylene glycol solution) and 2% xylocaine-hydrochloride (5% propylene glycol solution), large intensive blue spots soon appeared, which could not be observed with 0.03% of bufalin (5% propylene glycol solution) and 0.01% of gamabufotalin (5% propylene glycol solution).

As has already been described (1) some local anesthetic activity has been found in cinobufagin, cinobufatalin, gamabufotalin (Fig. 1a), etc., so that the local analgesic action of the Chinese crude drug, Ch'an Su, the toad venom, cannot be accredited entirely to bufalin. However, bufalin is the substance found until the present date that has the strongest local anesthetic action among bufadienolides, and a way will be opened for the use of steroid as a local analgesic by comparative examination of such substances to be prepared by changing their side chain.

Cinobufagin and cinobufatalin are about one-sixth of bufalin in local anesthetic potency, while resibufogenin has no such action. Digitoxigenin, a cardenolide possessing the same chemical structure as bufalin except for its 5-membered lactone ring, has a very weak local anesthetic action, about one-fortieth that of bufalin in surface anesthetic potency.

Cinobufaginic acid, formed by cleavage of the lactone ring through hydrolysis, was found to be entirely devoid of anesthetic action in the eye. The significance of the presence of unsaturated lactone ring, besides that of hydroxyl in C_3-position of steroid ring, seems to be essential for the local anesthetic action.

Bufalin has, however, a very weak anesthetic action on the nerve fiber as compared with procaine (Tables VIII and IX).

TABLE VIII

Compound	Threshold anesthetic concentration			Duration of anesthesia			Toxicity (mouse)	
	Sciatic nerve (frog) (%)	Cornea (rabbit) (%)	Intracutaneous wheal (human) (%)	Rabbit cornea 1% sol. (min.)	Human intracutaneous 0.5% sol. (min.)	Human intracutaneous 0.5% sol. + adrenalin 1:50,000 (min.)	S.C. LD_{50} (mg/kg)	I.V. LD_{50} (mg/kg)
Procaine	0.250	4.0	0.04	0-2	16	171	750	45
Butacaine	0.125	0.31	0.02	30	45	200	50*	12*
Dibucaine	0.004	0.003	0.0025	51 (0.1%)	100	221	10*	2.5*
Cocaine	0.125	0.32	0.02	22	12	200	110	11
Bufalin	—	0.0025	0.001	30 (0.02%)	30 (0.001%) 150-480 (0.01%)	—	4.0	0.35

* Rabbit

TABLE IX

Compound	Surface anesthetic potency (rabbit cornea)	ED_{50} (rabbit cornea)		LD_{50} (mg/kg) (mouse)		LD_{50}/ED_{50}	
		mg	%	I.V.	S.C.	I.V.	S.C.
Cocaine	1.0	0.85	0.437	11	110	12.9	129
Procaine	0.11	8.50	4.37	45	750	5.3	88
Xylocaine	0.27	3.16	1.74	31.5	172	9.9	54
Bufalin	88.2	0.01	0.06	0.35	4.0	29.2	400

The effect of bufalin and procaine on the electrical excitability of nerve fiber was examined, using the sciatic nerves of toads or bullfrogs. As described before (6), bufalin, in spite of its very strong potency in surface anesthesia, has only a weak effect on the nerve fiber. It was therefore supposed that the site of local anesthetic action of bufalin might be the receptor of sensory nerve ending. However, the site of action in question is now under detailed examination.

Bufadienolides as well as cardenolides induce a long-lasting contracture in the isolated frog *rectus abdominis* muscle, while cocaine, and other local anesthetics inhibit the acetylcholine contracture of the muscle. The muscular contracture curve consists of two stages: the stage of gradually increasing tonus, and the one inducing faster contracture following the former.

The second stage is inhibited by pretreatment with d-tubocurarine, as well as by tetrodotoxin, while the first stage is not affected by its pretreatment (presented in Fig. 3).

This contracture-inducing activity of bufadienolides and cardenolides runs parallel to the cardiotonic activity of these compounds.

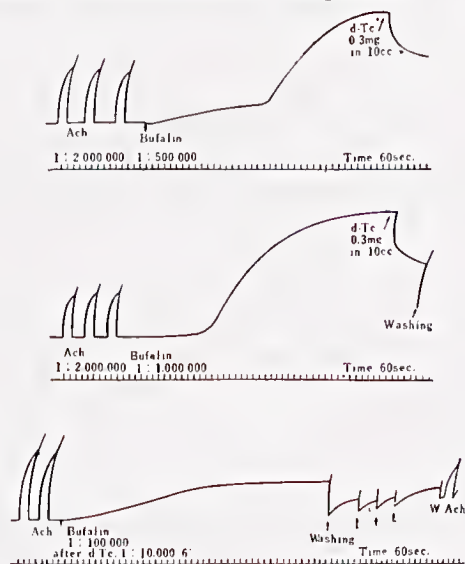



Fig. 3 — Bufalin on *Rectus Abdominis* Muscle of the Frog.

Method

(i) Sciatic nerve trunk was arranged in a separating box, as indicated in Fig. 4, stimulus was given to it at positions a and b (S), action-current was led off from positions d and e (R) and recorded. Drug was applied to desheathed part c. (ii) Direct stimulus was given to a single nerve fibre. As shown in Fig. 5, single medullated nerve fibre was excised, internode of N_1-N_2-N_3 was dried in the air, and N_2 was stimulated directly after application of drug to the node.

Fig. 4 — Experimental arrangement for nerve trunk. N: Nerve trunk. D.N: Desheathed part. S: Stimulus. R: Recording apparatus. a, b, e, d: Normal Ringer. C: Test solution.

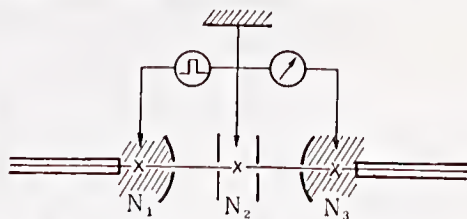



Fig. 5 — Experimental arrangement. $N_{1,2,3}$ Nodes exposed. $N_{1,3}$: in 0.3% Cocaine-Ringer. N_2: in test solution. N_1-N_2: Stimulating Circuit. N_2-N_3: Recording apparatus.

Action-current was conducted from space between N_2 and N_3 by means of a low input resistance (IM Ω) D.C. amplifier combined with a cathode ray oscilloscope, and recorded. Moreover, node N_1 and N_2 were previously anesthetized with 0.3% cocaine-Ringer solution. The stimulus was a square pulse of duration of 0.5 m sec. provided by a stimulator in (i) and (ii).

Thus, the aspect of decreasing action-current at applied region, together with the elapse of time, was observed.

Concentrations of the examined solutions were 2×10^{-4}, 1×10^{-4}, and 1×10^{-5} bufalin-Ringer's solution and 0.05-1.0% procaine-Ringer's solution, propylene glycol alone was examined at the same time.With the applied concentration of procaine-Ringer solution, action-current was found to decrease or disappear in nerve trunk and single nerve fibre. After the materials were brought to Ringer solution, and thus fully recovered, they were applied with bufalin.

With 1×10^{-5} concentration there was noted almost no effect but with 1×10^{-4} to 2×10^{-4} concentration there occurred reduction in action-current or inhibition on the conduction of excitation.

However, almost the same effect appears in propylene glycol itself with the same concentration as of the solution used as solvent, so that the action of bufalin on nerve trunk and single medullated nerve fibre may be far weaker than its surface anesthetic action on cornea, and strong local anesthetic action may be presumed to be due to its specific effect on the receptor of sensory nerve ending. Its mechanism is now under investigation.

As to the cardiotonic activity it has long been believed that the presence of OH groups in C_3- and C_{14}-positions was a requisite in cardenolides. In resibufogenin there is no OH group in C_{14}-position and there is a β-epoxide (α-epoxide is inactive) ring between C_{14}- and C_{15}-positions. In spite of this chemical structure, resibufogenin was found to have cardiotonic action nearly as strong as that of ouabain (Table X) besides marked activities in respiratory excitation

TABLE X — CONTRACTURE-INDUCING ACTIVITY OF THE VARIOUS CARDIO-ACTIVE STEROIDS AND THEIR DERIVATIVES

	Compound	Activity	Compound	Activity
(A)	Digitoxigenin	+ + +	Bufalin	+ + +
	Oleandrigenin	+ +	Bufotalin	+ + +
	Digitoxin	+ +	Resibufogenin	+ +
	Dihydrodigitoxin	+	Cinobufagin	+ + +
	Ouabain	+ +	Gamabufotalin	+ +
	Digitalinum verum monoacetate	+		
	Strospeside	+ +		
	Lanatoside C	+ +		
(B)	17 α-Digitoxigenin	—		
	β-Anhydrodigitoxigenin	—		
	14α, 15α-Epoxy-(β)-anhydrodigitoxigenin	—		
	17 α-Digitalinum verum monoacetate	—		
	β-Anhydrogamabufotalin	—		
	Deacetylcinobufaginic acid	—		
(C)	Tincture of digitalis purpurea leaf (pigeon LD50 74.0 mg/kg) effective in 10^{-3}			

+ + +	effective in doses of	$1\text{-}3\times10^{-7}$
+ +	"	$1\text{-}3\times10^{-6}$
+	"	1×10^{-5}
—	"	1×10^{-5}

and elevation of blood pressure notwithstanding its very low toxicity. This fact is considered to play an important role in further study of the cardiotonic action of these substances (Fig. 6, Tables XI-XII).

Cinobufagin

Resibufogenin

Bufalin

Cinobufotalin

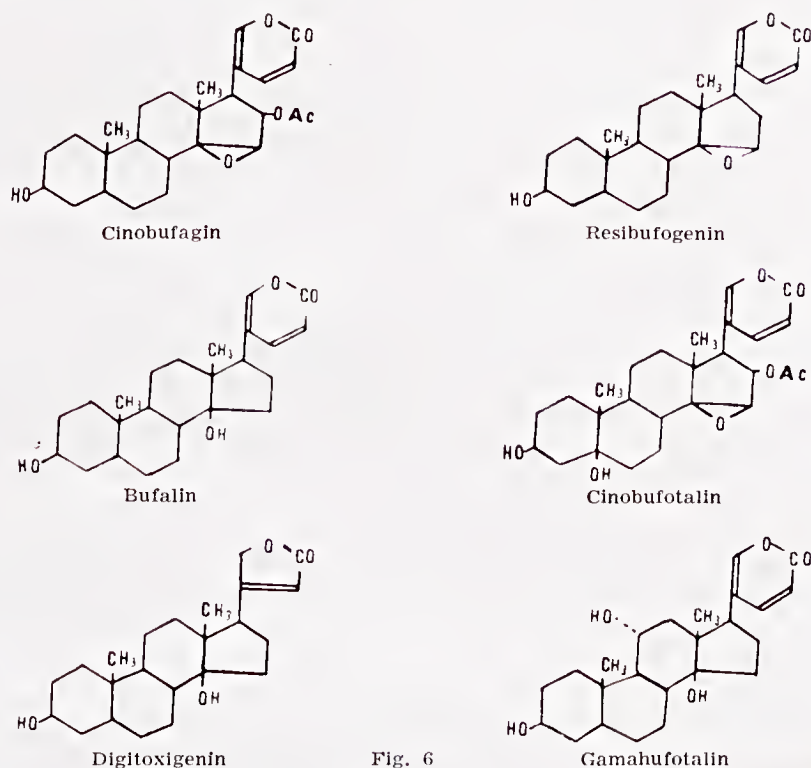
Digitoxigenin

Gamabufotalin

Fig. 6

TABLE XI — LETHAL DOSE OF BUFOGENINS AND ALLIED COMPOUNDS IN THE CAT (HATCHER-MAGNUS METHOD)

COMPOUND	Body weight (g) and sex of animal	Time of infusion (min.)	M.L.D. (mg/kg)	M.L.D. reported by other workers (mg/kg)
Bufalin	2680	47	0.157	0.137
Gamabufotalin	2000	12	0.134	0.101
	2400	30	0.137	
Cinobufagin	2700	54	0.296	0.200
Resibufogenin	2350	110	3.2	5
Digitoxigenin	1960	49	0.411	0.450
Digitoxin	3450	130	0.365	0.325
Strophanthin	2400	64	0.118	0.116

TABLE XII — FROG SYSTOLIC STANDSTILL METHOD (hr.)

COMPOUND	ED_{50} (μg/g)	95% Confidence limit (μg/g)
Bufalin	1.01	0.95-1.08
Gamabufotalin	1.35	1.26-1.44
Resibufogenin	7.11	5.29-8.93
Strophanthin	0.28	0.24-0.33
Digitoxin	1.75	1.62-1.89
Digitoxigenin	6.10	4.80-7.75

On this mechanism of respiratory excitation of resibufogenin the following experiment was made on urethanized rabbit. Even after intravenous injection of 10 mg/kg of procaine, which inhibits the carotid sinus reflex, and causes complete disappearance of respiratory-stimulating action by 0.05 mg/kg nicotine tartrate and 0.3 mg/kg lobeline hydrochloride, the respiratory stimulating action of resibufogenin could be observed, and it was found to be almost equal to that induced by single administration of the same substance. Moreover, respiratory excitation by nicotine disappeared completely due to removal of carotid sinus nerve and nodular ganglion, but resibufogenin revealed a remarkable respiratory stimulating effect even after the same operation. The respiratory excitation by resibufogenin, being different from the reflective one by nicotine and lobeline through the carotid sinus, is supposed to result from its direct action on the respiratory center.

As for the mechanism on blood pressure, intravenous injection of 5-10 mg/kg of hexamethonium in urethanized rabbit inhibited the action of picrotoxin on the elevation of blood pressure, but had almost no effect on the same action of resibufogenin. In spinal cat, the action of picrotoxin disappeared completely, but resibufogenin revealed a distinct effect. Even after administration of 10 mg/kg of Priscol and 5 mg/kg of chlorpromazine the action of resibufogenin on the elevation of blood pressure was noted to be almost similar to that by single administration of the same substance. Moreover, resibufogenin caused considerable elevation of blood pressure even when it was previously lowered by intravenous injection of 5 mg/kg of $NaNO_2$. This fact suggests that resibufogenin affects blood pressure peripherally differing from picrotoxin which acts centrally only.

On the other hand bufotenidine, the betain of bufotenine, of the water-soluble fraction of Senso, was found to have an action which closely resembles that of nicotine though far more potent than the latter.

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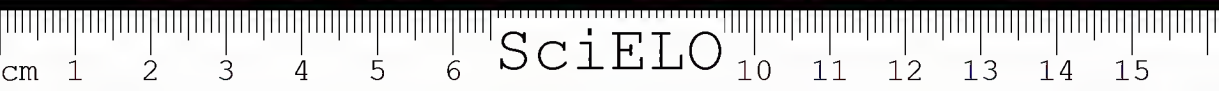
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Discussion

F. Kornalik: "Have you observed any other local reactions such as vasoconstriction or any other effects upon the cardiovascular system in locally anesthetic doses of bufalin?"

M. Okada: "Bufalin and others have weak vasoconstrictory actions, but they have almost no other actions in the concentration as they are used as local anesthetics."

Mem. Inst. Butantan
Simp. Internac.
33(2):603-614, 1966

62. SOME ASPECTS OF THE PHARMACOLOGY OF THE VENOMS OF AFRICAN SCORPIONS

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Introduction

The scope of this review is confined to work carried out, mainly in recent years, on the venoms of African scorpions, and may thus include such species as *Androctonus australis, Buthus occitanus, Leiurus quinquestriatus*, and *Buthotus minax*, but in the present context, unless otherwise stated, the term "venom" will refer to the venom of *Leiurus quinquestriatus*. The pharmacological studies mentioned have all been carried on vertebrate tissues.

Most commonly, the venoms have been obtained by electrical stimulation of the telson and have been promptly dried. It is generally agreed that, in the dry state, the venoms of these species preserve their toxicities for months or years (1, 2, 3). Most reports have stated that the toxic factors are non- or poorly-dialysable, but recent studies by Shulov and his colleagues (4, 5) claim that there is a dialysable factor in fresh venom which is toxic to mice. It is, therefore, possible that the common use of dried or freeze-dried venom has obscured the existence of another toxic substance and further work should be devoted to this point.

It is probably true that the fraction of lyophilized venoms of the above species, which is toxic to vertebrate tissues, consists of one or two basic proteins. The evidence for this was originally based on electrophoretic studies, dialysis, and inactivation by proteolytic enzymes (2, 3) and has been substantiated the work of Miranda and his colleagues at Marseilles on the nature of the toxic components of *A. australis* and *B. occitanus* venoms (6, 7, 8). In our experience, it would appear that the toxic constituents in *L. quinquestriatus* venom bear a strong resemblance to those studied by Miranda *et al.*, but unfortunately we have not been so successful in obtaining clear separations of the two basic proteins in *L. quinquestriatus* venom thus far. The use of electrophoretic eluates indicated that neuromuscular was associated mainly with the slower-moving of the two protein bands, which is interesting as Miranda and Lissitzky (9) found that the two basic proteins in *A. australis* venom shared almost equally the toxicity to mice. In our case, insufficient material was available in the eluates for toxicity testing; it is clearly desirable that pure protein fractions should become available and enable us to be more precise in allocating pharmacological effects to individual components.

In the actions to be discussed next, the fraction of lyophilized venom precipitated by 80% (v/v) acetone at −15°C was used, and contained both of the basic proteins seen on electrophoresis.

NEUROMUSCULAR ACTIONS: Considerable interest centres on the neurotoxic activity of venoms. One of the most obvious effects of scorpion venom is to provoke twitching and fibrillation of skeletal muscle, and it is natural to inquire as to the site or sites at which this effect takes place. Houssay (10) showed that the application of venom to a motor nerve could cause twitching of the innervated muscle, but he considered that nerve was less sensitive than muscle because he had to apply much higher concentrations of venom to nerve in order to obtain an effect. However, if one de-sheaths part of the nerve, then that part becomes at least as sensitive to venom as is the muscle, indicating that the epineurium forms a considerable barrier to the venom molecules (11).

To analyse the effect on nerve more closely, it is advantageous to use isolated nerve fibres, perfusing a Ranvier node with venom solution and noting the effect on the electrical activity. This has been done recently (12). With low
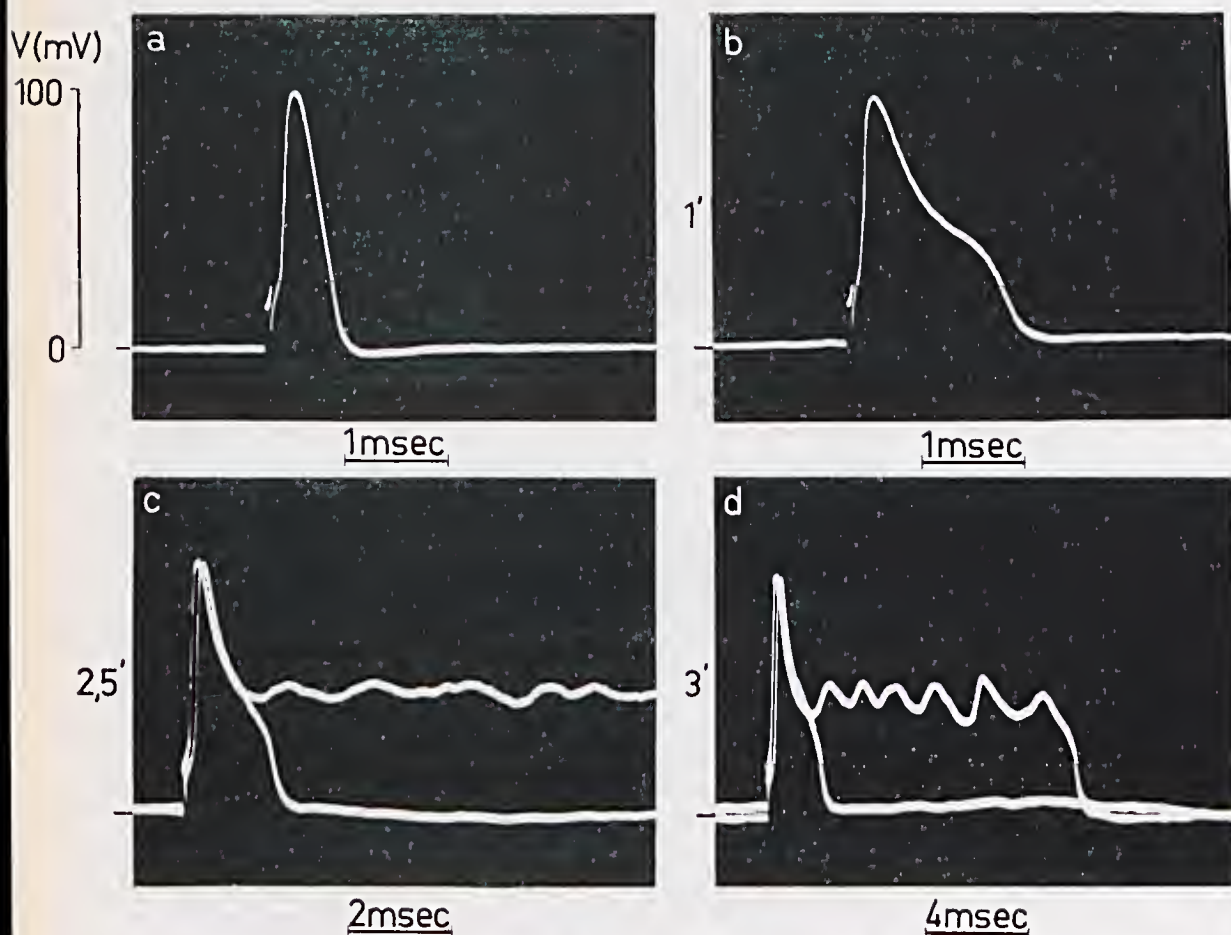
Fig. 1 — Action potentials of a sensory fibre in normal Ringer solution then 1, 2.5, and 3 minutes after addition of venom (5×10^{-7}). Node stimulated once per second.

concentrations of venom (5×10^{-7} — 5×10^{-8}) the most striking effect is the prolongation of action potentials (Fig. 1). These increased in duration from the normal one msec. or so up to a second or more, and occasionally lasted as long as ten seconds. Since, initially, the rising phase of the action potential was unaltered, it seemed probable that the fast increase in Na permeability was unaffected. The falling phase of the normal action potential is due to a combined change of Na and K permeabilities, but as no effect on delayed rectification was found it seemed unlikely that the venom affected K permeability. Therefore it was considered that the observed prolongation of the action potential was due mainly to delay in the inactivation of Na permeability. This view was supported by voltage clamp experiments.

With higher concentrations of venom ($> 5\times10^{-7}$) and longer exposure, depolarization and spontaneous action potentials occurred (Fig. 2). Perfusion of
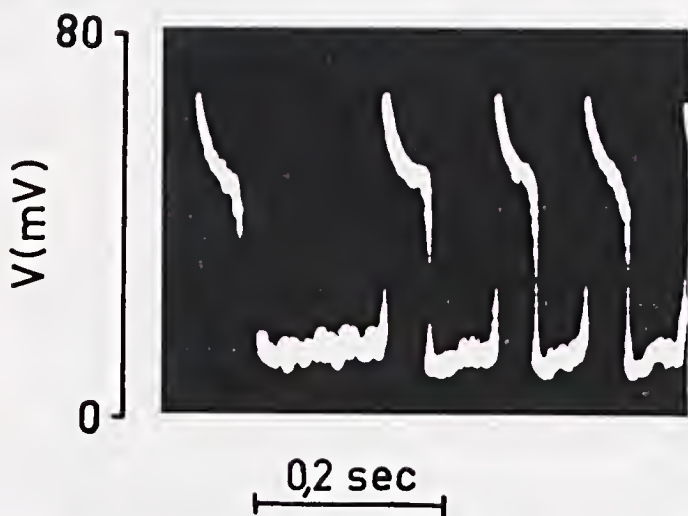
Fig. 2 — Spontaneous activity of a motor fibre superfused with Ringer containing venom (5×10^{-7}). Photograph taken after 18 minutes exposure to venom.

the node with low-sodium solutions abolished the effect of venom, and increasing the sodium to normal Ringer levels restored the effect again. Generally, the action of venom on the resting potential was strongly dependent on the concentrations of sodium, the depolarization amplitude rising with increasing sodium concentration at a rate suggesting that the resting membrane was rendered much more permeable to Na by the venom (Fig. 3).

The venom-induced depolarizations and the spontaneous firing of impulses in the nerve fibres of a nerve-muscle preparation will, of course, initiate twitching of the muscle. Is there evidence for a direct action on muscle as well? A contracture of isolated skeletal muscle can be obtained with venom in the presence of sufficient tubocurarine to antagonize quite large quantities of acetylcholine (2). In Fig. 4, a rat diaphragm is being stimulated directly (submaximally) in the presence of tubocurarine. At the arrow 5 μg venom/ml was added, resulting in a contracture and increased amplitude of the twitches. Gradually, the effect diminishes as time goes on, and over the next 15-30 minutes
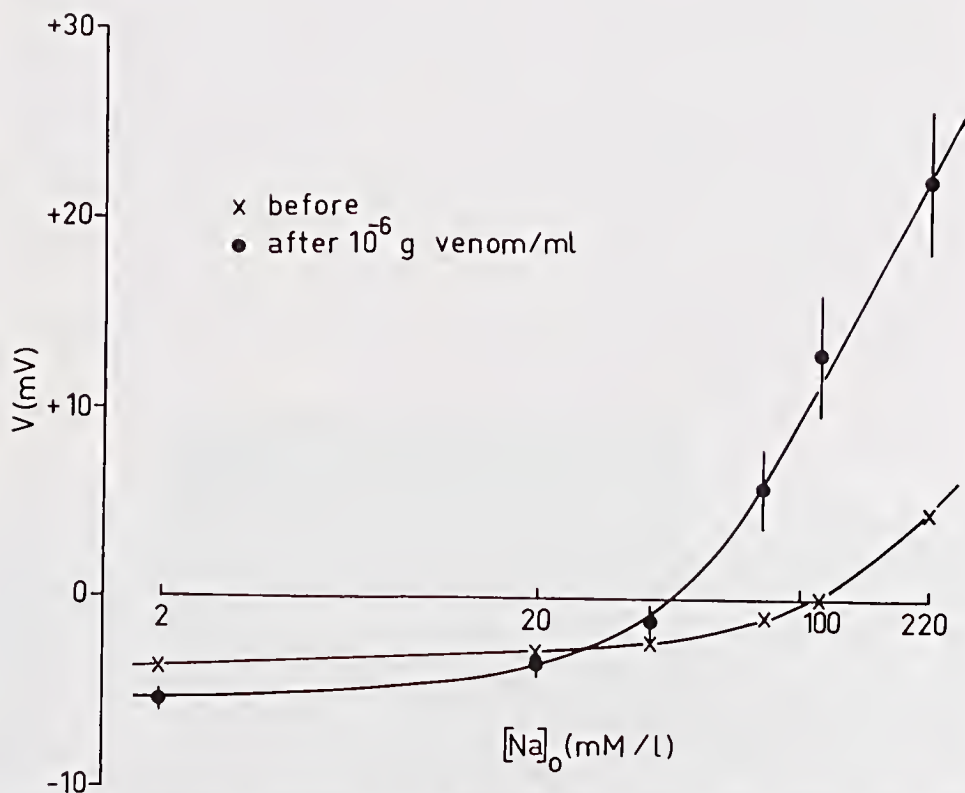
Fig. 3 — Relationship between Na concentration of the medium and membrane potential before and after exposure to venom (10^{-6}). Ordinate, change of membrane potential in mV (depolarization positive). V = 0 is the normal resting potential before application of venom. Mean values and standard errors of the means of 14 fibres.

the twitch amplitude decreases until the muscle becomes almost inexcitable. With higher concentrations of venom, violent spontaneous twitches are superimposed initially.

It seems likely, therefore, that there is a direct effect on skeletal muscle and, inkeeping with this, records resting membrane potentials from Sartorius fibres show a slow depolarization under the influence of venom until an action potential is elicited (Adam and Weiss, unpublished observations). Possibly this, too, may be due to an effect on Na conductance through the membrane but, as yet, there is no experimental evidence. Thus the effect obtained with venom on an isolated or *in situ* nerve-muscle preparation is probably due to an action on both muscle and nerve, though it seems likely that the latter is affected more readily.

Comparison with veratrine: Since the early work of Houssay (10), the effects of several venoms on nerve and skeletal muscle have been noted to resemble those of veratrine or one of its constituent alkaloids, veratridine. Fig. 5 shows the effect of 35 μg veratrine/ml on the directly, submaximally, stimulated rat diaphragm, inducing a contracture and increased twitch amplitude very similar to that shown in Fig. 4. Various differences, e.g. the smaller effect of
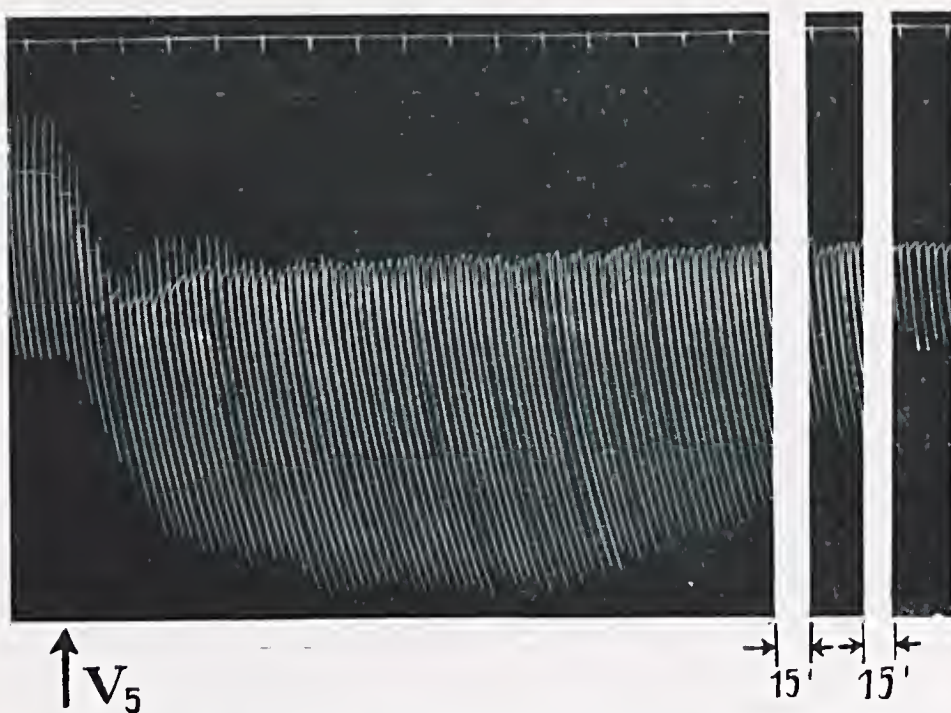
Fig. 4 — Rat diaphragm. Direct submaximal stimulation. 5 μg venom/ml added at arrow. Between the last two records 15 minutes elapsed.
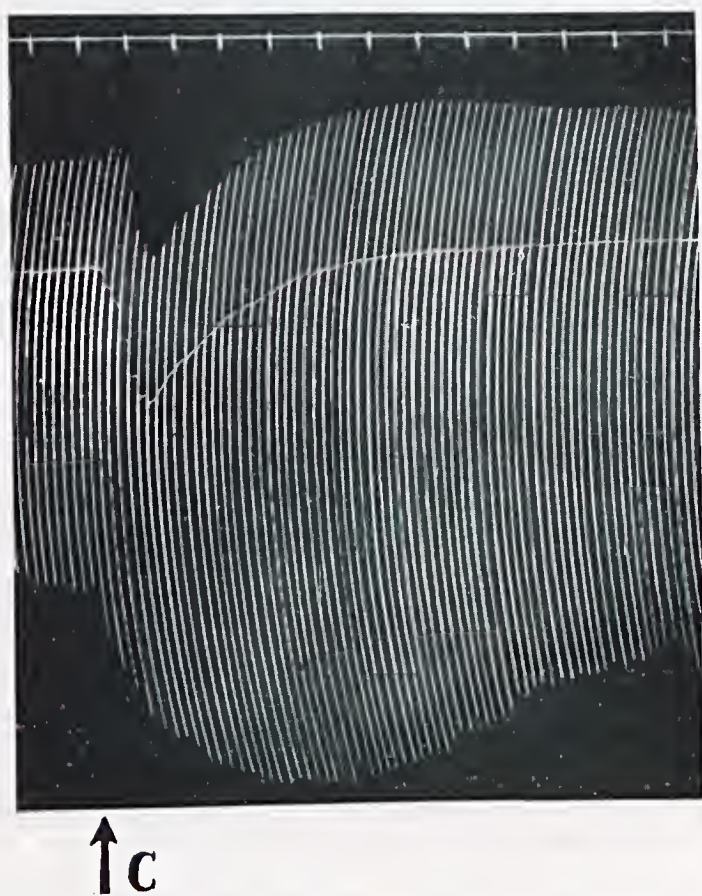
Fig. 5 — Rat diaphragm. Direct submaximal stimulation. 35 μg veratrine/ml added at arrow.

veratrine on muscle relaxation after a twitch, between venom and veratrine can be demonstrated (2, 12) but on the whole there is a striking similarity. Veratrine has been shown to have direct depolarizing effects on skeletal muscle and on nerve fibres, and these effects have been interpreted as due to increases in membrane permeability to Na (13, 14, 15, 16). Thus the observed similarity in the actions of venom and of veratrine may have its basis in a common mechanism. Again, the effects of both venom and veratrine resemble those induced by low-calcium solutions and by Ca-chelating agents, and can be at least partially antagonized by increasing the calcium concentration of the medium (2, 16, 12). Such effects have been demonstrated with various venoms for many years (17). Displacement of Ca from membranes was suggested as the mechanism of veratrine action by Gordon and Welsh (18) and it seems possible that venoms could displace Ca in a similar way. One might speculate that the positively-charged venom molecules display an affinity for the membrane acidic phospholipids in preference to Ca or to Na, thus interfering with a Na-carrier system, but it is probably not profitable to pursue further any discussion of Ca-venom antagonism at the membrane at present. However, it should be remembered that various membrane "stabilizers", such as adrenaline or local anaesthetics, can be shown to block or reverse phases of venom action, and it has been postulated that their stabilizing effects may be mediated via the membrane Ca (19, 20, 21).

Pain production by venom: This is another notable effect of scorpion venom, and may well be related to the actions on nerve fibres discussed previously. In some cases it is possible that the presence of large quantities of serotonin may contribute to the pain of a sting (22, 23) but probably the major pain-producing substance is protein. Using the Armstrong-Keele cantharidin blister technique (24), we found that venom could still cause pain when its serotonin content was below threshold; that pain could be provoked by the application of eluates from a protein band after electrophoresis; and that it was usually possible to detect a qualitative difference between the pain produced by pure serotonin and that due to venom (Adam, Smith & Weiss, unpublished observations). It seems possible that the same substances which provoke neuromuscular activity are responsible for most of the pain of a sting, and that this may be due to a direct stimulation of sensory nerve fibres by the mechanism already discussed. It is interesting to note that both veratrine and citrate can induce pain at the blister base (25).

Phospholipase A activity: Although *L. quinquestriatus* venom has been shown to have some haemolytic activity (26), it does not appear to contain phospholipase A. Incubation of venom with purified phospholipid substrates, human plasma, rat-brain or rat-muscle homogenates results in a negligible increase in free fatty acids, and a lack of degradation of di-acylphospholipids can be confirmed chromatographically (27). One wonders if there is a direct lytic factor and whether this might be the neurotoxic protein interfering with cation transport across the erythrocyte membrane. However, there is some evidence that the toxicities of various venoms do not parallel their haemolytic activities (28).

To summarize: It seems likely that the main action of *L. quinquestriatus* venom is to interfere with the mechanism switching on and off the Na permeability of cell membranes. While this is a suitable working hypothesis for current pharmacological studies, it is evident that the immediate requirement for further advances is the provision of pure venom fractions in adequate amounts.

Acknowledgment — One of the authors (K.R.A.) wishes to acknowledge his indebtedness to the Welcome Trust for a travel grant enabling him to attend the Symposium.

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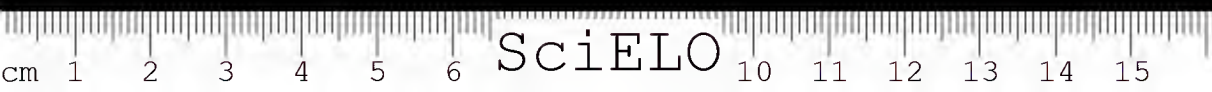
Discussion

O. Vital Brazil (Department of Pharmacology, University of Campinas, Campinas, São Paulo: "Although it was known since the investigations of Maurano (1915), Vital Brazil (1918) and Houssay (1919) that the venom from the South American scorpions belonging to the genus *Tityus* was immunologically different from that of the African scorpions, their pharmacological actions were believed to be almost identical. However, by comparing the results obtained by Prof. Adam employing the venom of *Leiurus quinquestriatus* and that of other Buthinae, with our own results using the venom of *Tityus serrulatus*, we can now appreciate that there are also important pharmacological differences between these venoms. In fact, the venom of *T. serrulatus* never produced, in our experiments, the contracture of the isolated rat diaphragm as described by Prof. Adam for the venoms of the African Buthinae.
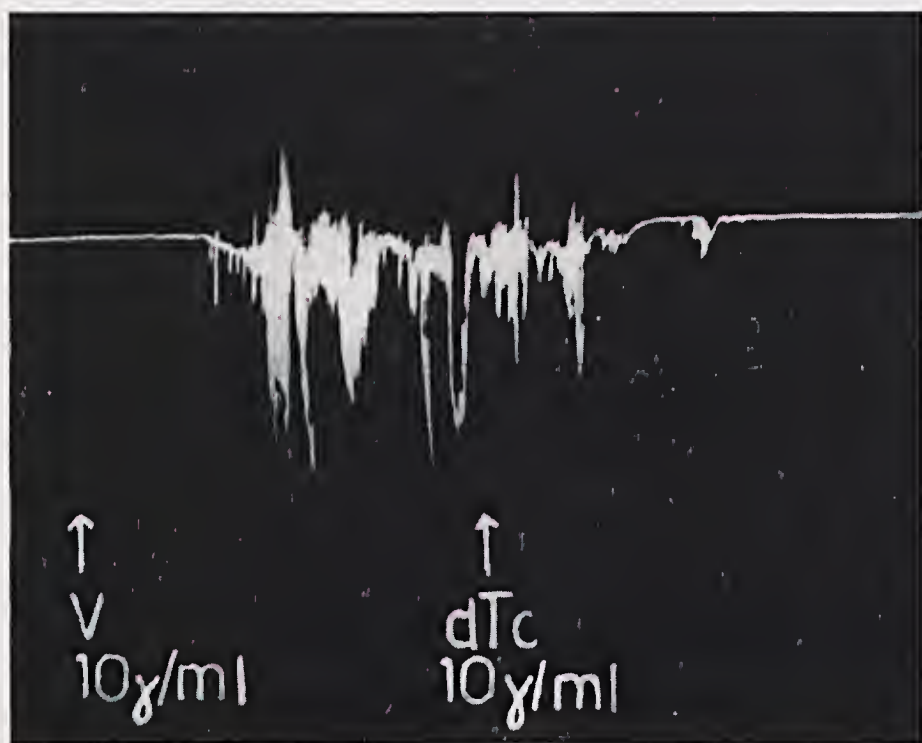
Fig. 1 — Isolated phrenic nerve-diaphragm preparation of the rat. The venom (10 mcg/ml) of *Tityus serrulatus* evoked contractions of the diaphragm which were abolished by d-tubocurarine (10 mcg/ml).

Instead, it produced intense twitchings of the muscle which were promptly abolished by d-tubocurarine (Fig. 1). Therefore, the venom of *Tityus serrulatus* seems to be devoid of the direct muscular action exhibited by the venoms of the African Buthinae.

The twitchings as well as the increase caused by *T. serrulatus* venom in the amplitude of the response evoked by isolated supramaximal shocks applied to the nerve (Fig. 2), can be explained by acetylcholine release from the motor nerve terminals. Such a release of acetylcholine caused by the venom has recently been demonstrated in my laboratory at the University of Campinas. The isolated inervated, and sometimes the isolated chronically denervated hemi-diaphragm, were
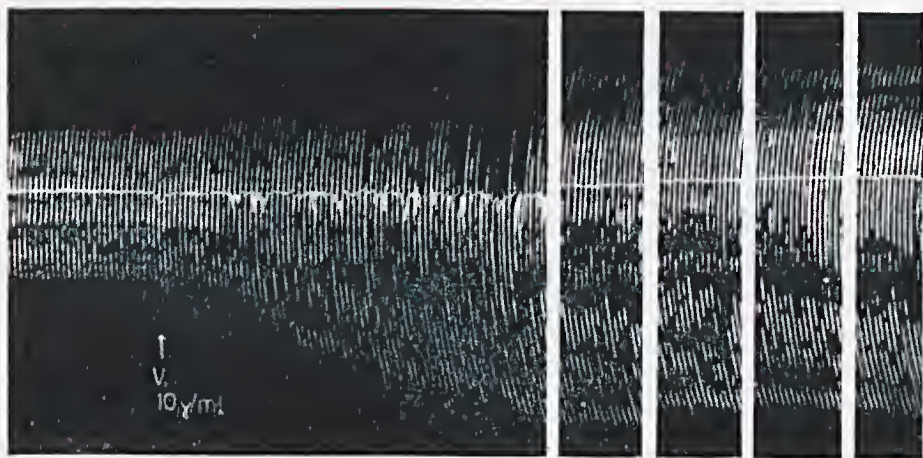
Fig. 2 — Isolated phrenic nerve-diaphragm preparation of the rat. The venom of *T. serrulatus* caused a great increase in the responses produced by nerve stimulation with supramaximal shocks delivered at a rate of 6 per minute.

used in these experiments. They were suspended in Tyrode solution containing 0.2 per cent of glucose and 5×10^{-6} of neostigmine methylsulphate. The bath volume was 5 ml and its temperature, 37°C. The preparations were oxygenated by bubbling a mixture of 95 per cent O_2 and 5 per cent CO_2. The fluids after bathing the diaphragm for 20 minutes were removed and immediately assayed for acetylcholine by its depressor effect on the arterial blood pressure of anaesthetized small cats injected with hexamethonium and ephedrine. The results can be summarized as follows:

1. A very small spontaneous release of acetylcholine sometimes occurred, the acetylcholine content of the fluid being always less than 0.4 ng per 0.2 ml. Therefore, the spontaneous release of acetylcholine by the hemi-diaphragm was always less than 10 ng.

2. The venom promoted the release of acetylcholine from the inervated hemi-diaphragm. The acetylcholine content of the venom containing fluids which bathed the hemi-diaphragm for 20 minutes was seldom less than 2 ng per 0.2 ml; in most instances it varied from 2 to 4 ng per 0.2 ml (Fig. 3). Therefore, the acetylcholine chlorine released by the venom from the hemi-diaphragms could be estimated to be from 37.5 to 100 ng.

3. The venom did not release acetylcholine from chronically denervated hemi-diaphragms.

4. Curarization of the hemi-diaphragms with d-tubocurarine did not seem to reduce the release of acetylcholine by the venom.

5. The venom did not released acetylcholine when the fluid bathing the hemi-diaphragm contained procaine.

6. The acetylcholine released by venom seemed to be calcium dependent. When this ion was suppressed from the Tyrode solution no acetylcholine release could be demonstrated. When it was increased above the usual concentration in the Tyrode solution, an increase in the acetylcholine output was verified.

The mechanism of acetylcholine release by the venom is unknown. Depolarization of the nerve fibres by the venom as Prof. Adam has verified, would explain it."
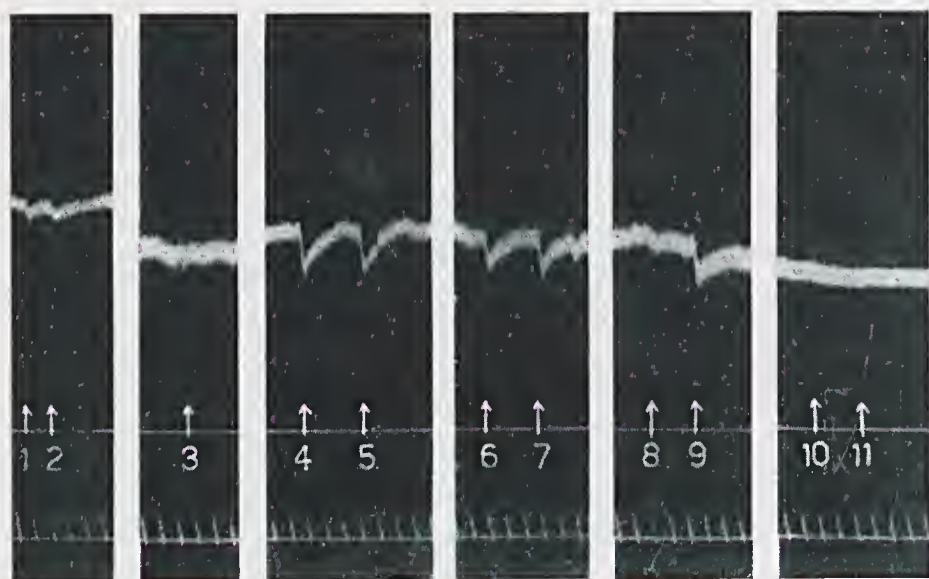
Fig. 3 — Arterial blood pressure of the cat. 1. and 2. — 0.2 ml of Tyrode solution with neostigmine which bathed the diaphragm for 20 minutes (spontaneous release of acetylcholine); 3. — 0.2 ml of Tyrode solution with neostigmine containing 20 mcg/ml of *T. serrulatus* venom; 4. and 5. — 0.2 ml of Tyrode solution with neostigmine containing 20 mcg/ml of *T. serrulatus* venom after bathing the diaphragm for 20 minutes (acetylcholine released by the venom); 6. — 4 ng of acetylcholine chlorine; 7. — the same as 4. and 5.; 8. — spontaneous release of acetylcholine after Tyrode solution with neostigmine containing the venom was removed and the diaphragm washed; 9. — the same 4. and 5; 10. and 11. — acetylcholine (4 ng) and acetylcholine released by the venom (0.2 ml) after the injection of 2 mg/kg of sulphate of atropine. Cat anaesthetized by pentobarbital (30 mg/kg, i.v.) and injected with hexamethonium bromide and ephedrine sulphate.

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Discussion

A. Shulov: "1. Whether are there any differences in results obtained through use of fresh and lyophilised venom? 2. Whether any experiments were carried out with scorpion venom such as from *Scorpio maurus* occurring in Sudan?"

K. R. Adams: "We have no evidence regarding the first question, but have this point very much in mind. We have not had the opportunity of investigating the venom of *Scorpio maurus*."

P. Efrati: "I was deeply interested in the observations presented by Prof. Adam. My experience concerns, unfortunately, human beings stung by *Leiurus quinquestriatus*. Besides of pains, observed predominantly in adults, we have observed

symptoms in children, which could be explained by discharge of diencephalic centers: sympathetic, parasympathetic manifestations as well as manifestations of integrating centers, for instance, those involved in thermoregulation: chill, piloerection, hyperthermy, perspiration. I would like to know, how your basic observations could be applied to the understanding of the clinical syndrome?"

K. R. Adam: "There is undoubtedly considerable autonomic discharge as the result of a sting. This has been shown by several authors. There would seem to be no reason for autonomic fibres being exempted from the stimulation that occurs to sensory and motor fibres, as presumably the venom molecules can gain access to them."

P. Krag: "Could the method for scorpion venom on nerve fibres be used for testing antibody — to prevent venom action or to stop the action already initiated — of course with due regard to Ca^{++}- and Na^{+}-concentrations?"

K. R. Adam: "This is a new idea to me. It would seem to be a possibility."

H. I. Bicher: "May part of the mechanism be related to a change in K^{+} permeability instead of Na^{+}? The resemblance of this record with those obtained when Ba^{+} is applied in similar systems makes me consider this possibility."

K. R. Adam: "The lack of any effect on the delayed rectification suggested little action on K^{+} permeability, and the prolonged effect on Na^{+} permeability in voltage clamp experiments seemed adequate to explain the prolonged action potentials."

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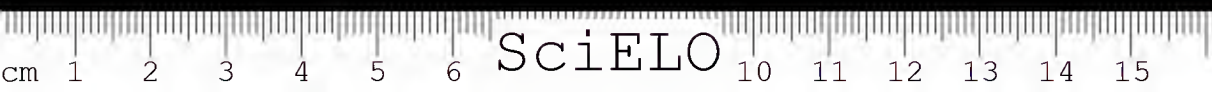
63. PHARMACOLOGY OF THE VENOMS OF MEXICAN *CENTRUROIDES*

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Instituto de Salubridad y Enfermedades Tropicales, México

Recent reports from electrophysiological and biochemical work seem to show qualitative differences in scorpion venoms. Old descriptions of both clinical and experimental poisoning are similar and only quantitative discrepancies are found in observations corresponding to a wide variety of species from distant parts of the world. Differences in symptoms or signs of intoxication seemed to be due to variable amounts of venom or different content of active principles (1).

The general actions: muscular twitchings, ptyalism, progressive respiratory irregularities reaching respiratory paralysis in some cases, pilo-erection, mydriasis, blood pressure rise, signs of local pain, signs of laringeal constriction, are to be found in accounts of scorpion poisoning from any part of the world. However, the mechanism invoked to explain such actions varied very much.

At present, fundamental work is being done with scorpion venoms in many laboratories and agreement seems to be reached about the effects on nervous system and neuromuscular junctions. Nevertheless new discrepancies could be found in reports from electrophysiological studies. Different techniques are being used and one should keep in mind that discrepancies are early and stimulating aspects of original research.

In spite of the fact that new paths of research in pharmacology of scorpion venoms may lead to the discovery of a common mechanism of action, it is essential to mention always the scorpion species used in each work.

The description that follows is limited to pharmacological properties of venom from Mexican *Centruroides*, and it is based on works from my laboratory. Our comparative studies of the actions of venoms of *C. suffusus suffusus* Pocock, *C. noxius* Hoffmann, *C. limpidus tecomanus* Hoffmann and *C. limpidus limpidus* Karsch, grant a common consideration. Only quantitative differences have been found. Larger doses of the less active venoms reproduce the effects of smaller doses of the more active ones.

MUSCULAR EFFECTS — One of the most immediate effects of intravenous injections of scorpion venom in cats, dogs, mice and rats is the appearance of generalized muscular twitchings and fascicular contractions. This activity originates in the spinal cord. It persists after section of the brain stem, deafferentation or transection of the spinal cord. The fascicular contractions below the level of the section are more marked than above, but they disappear completely when the spinal cord is destroyed or the motor nerves are cut (Fig. 1).
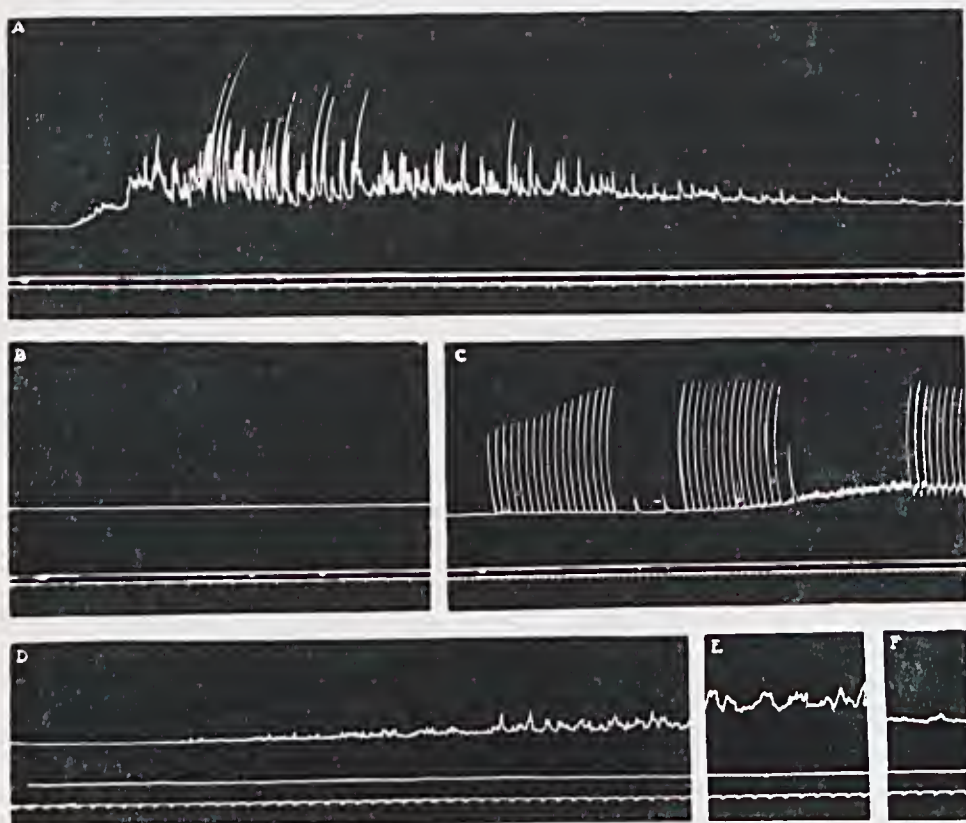
Fig. 1 — A: Muscular effects of an intravenous injection of scorpion venom. B: No effect is obtained when an equal amount of venom is injected after the muscular nerve was cut. (This and Figures 3 to 7 taken from del Pozo and Anguiano (2)).

Muscular twitchings and fibrillations are also obtained if the venom is applied directly to one muscle or injected intrarterially even with the corresponding nerve cut. This muscular activity does not occur when time has been allowed for Wallerian degeneration after denervation. Local application of venom to a muscular nerve trunk does not produce any effects on the muscles (2).

The local action of the venom takes place on the muscular end-plate region. This inference was confirmed when electrical records were taken from muscle and nerve. Repetitive potentials were found following single shocks applied to the nerve centrally cut. This repetitive activity is conducted antidromically in the nerve and disappears when this nerve is disconnected from the muscle. When records were taken from anterior and dorsal roots, the repetitive activity was seen in the former and not in the latter (4) (Fig. 2).

Big and long-lasting contractions are obtained when single shocks are applied to muscles under the action of scorpion venom. When successive responses are provoked at short intervals, the contractions are progressively smaller in amplitude and duration. Electrical recordings show at the same time a gradual diminution of the repetitive activity (Figs. 3 and 4).

According to this evidence the great and long muscular responses to single shocks correspond to short tetanic contractions consecutive to the repetitive activity provoked at the end-plate regions of poisoned muscles by the arrival of single impulses.

These effects on time and duration of muscular contraction and the influence of repetitive stimulation have been extensively analyzed at different frequencies of stimulation. The graphs of tension development of the tetanic contractions shows mechanograms resembling those obtained when higher frequencies of stimulation are applied to normal muscles (2) (Fig. 5).
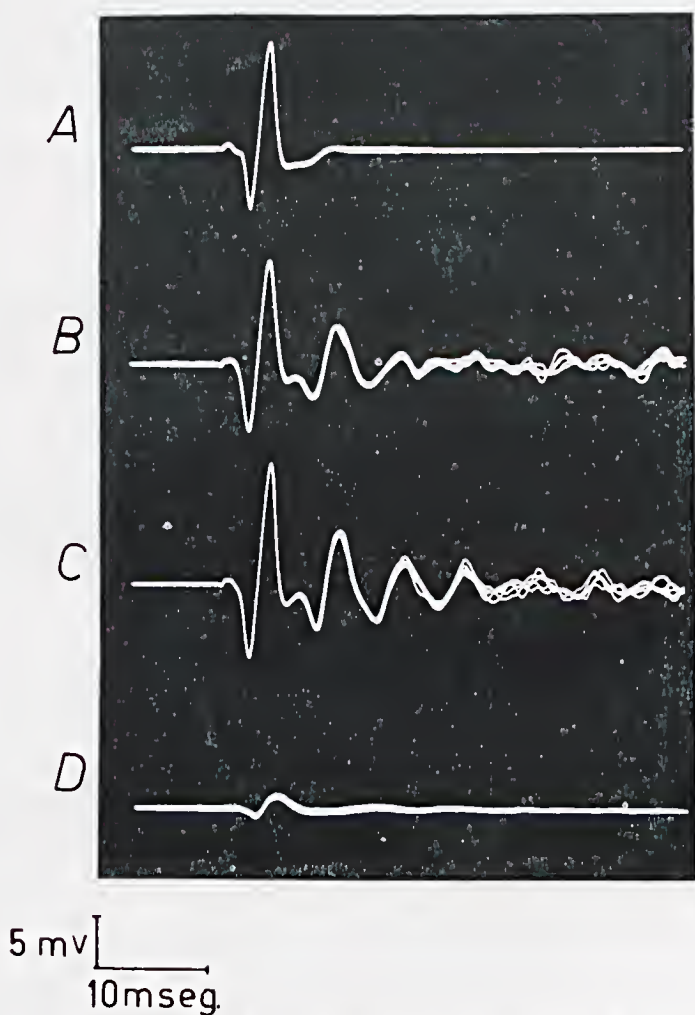
Fig. 2 — Electrical recording from gastrocnemius muscle of the cat. Five superimposed responses to shocks applied to the nerve in each segment. A, before; B to D, after successive dosis of scorpion venom. (This and fig. 8 taken from del Pozo, Salas and Pacheco, in press).
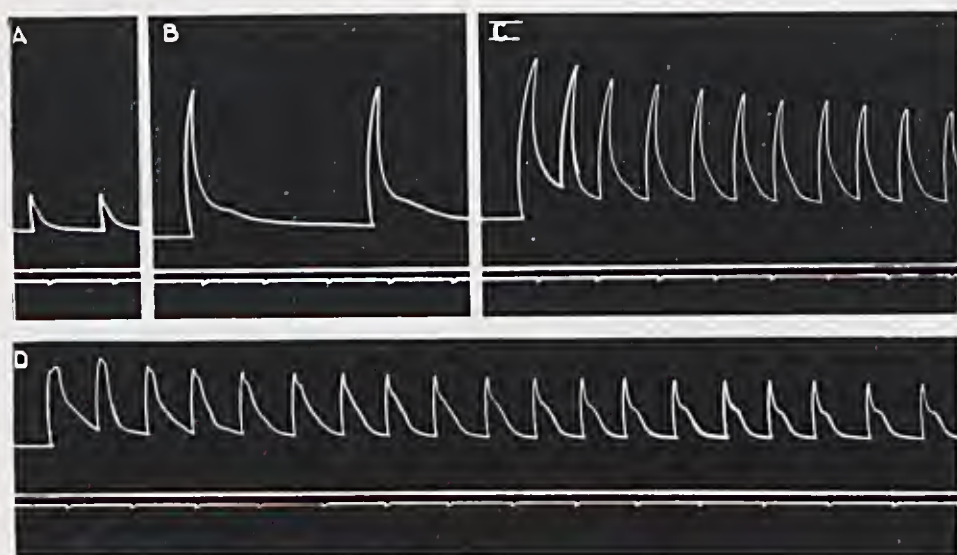
Fig. 3 — Effect of scorpion venom on the amplitude and duration of muscular responses. Maximal contractions of gastrocnemius of the cat, before (A) and after the injection of the venom (B). In successive contractions, the amplitude and duration are progressively reduced (C). (D), 20 minutes later.
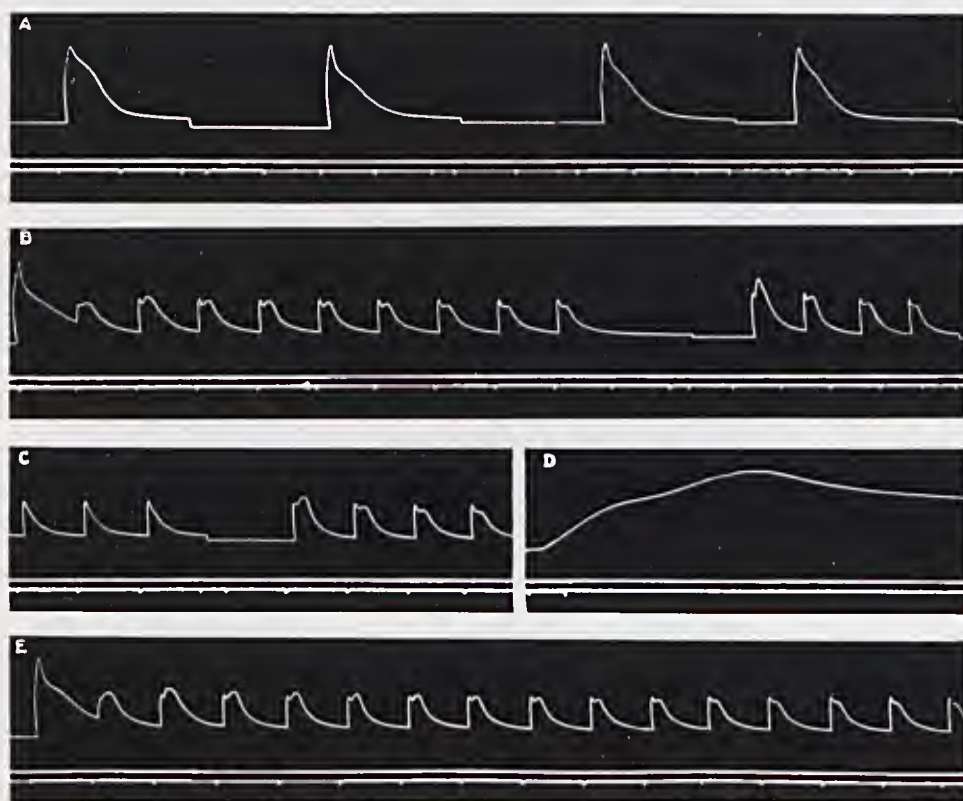
Fig. 4 — The length of the interval between stimulations change the type of responses in a muscle under the action of scorpion venom. A, 3 minutes interval between shocks. B, one second intervals.
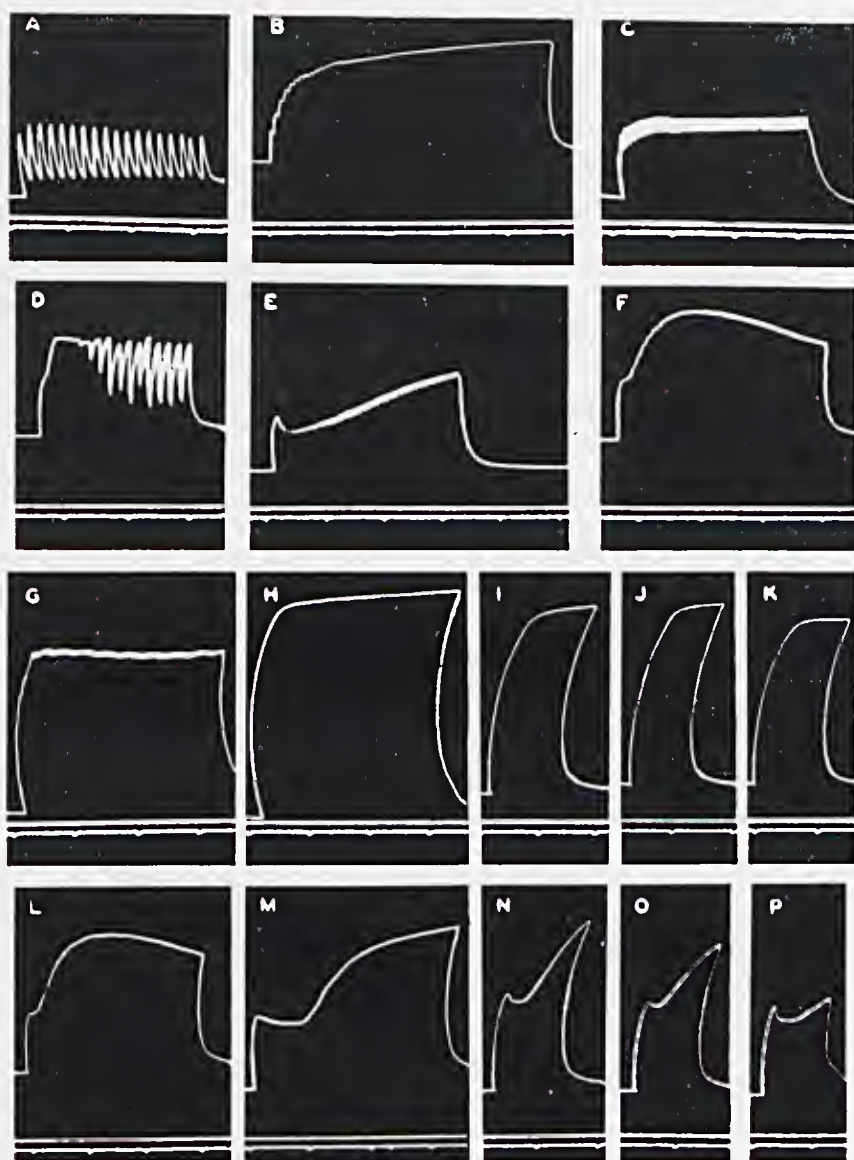
Fig. 5 — Effect of scorpion venom on muscular responses to high frequency stimulation. Pairs of responses before and after injection of the venom to stimulation to the following frequencies per second: A and D, 5; B and E, 13; C and F, 17; G and L, 25; H and M, 30; I and N, 60; J and O, 120; and K and P, 200. Gastrocnemius of the cat.

The tetanizing minimal frequency of the stimulus for one particular muscle is smaller when poisoned than for a normal one, but after the initial complete tetanus the individual responses to each shock appear on the graph. This peculiar development of the contraction is the opposite of the normal graph obtained at the minimal tetanizing frequency, i.e., initial individual response that progressively

fuse in a complete tetanus. The long lasting initial contractions of the poisoned muscle and the progressive shortening of the successive responses account for this phenomenon (Fig. 6).
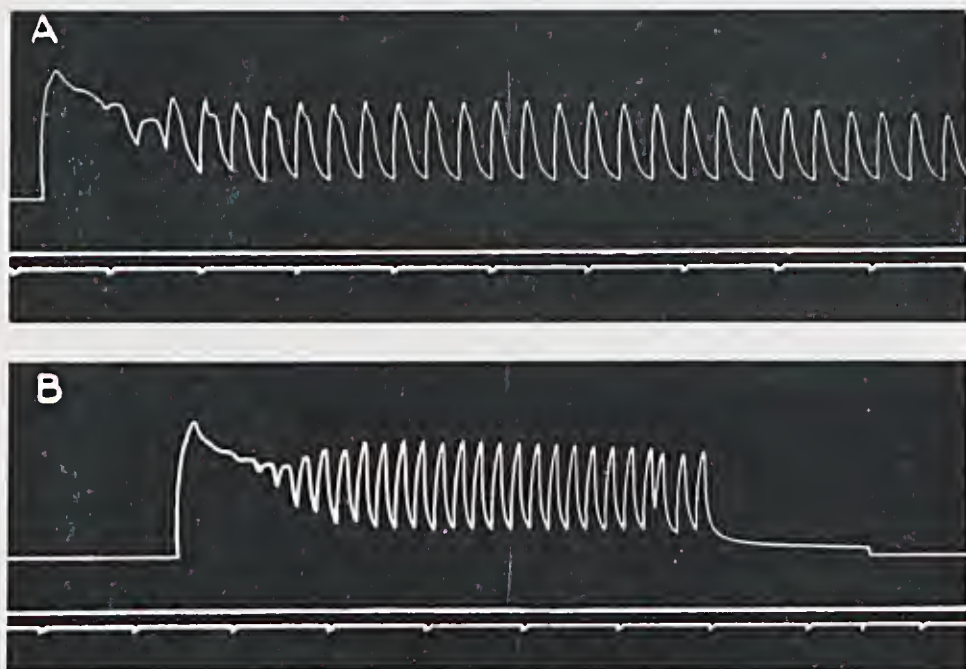
Fig. 6 — Inversion of the stages of the responses to stimulation at a minimal tetanizing frequency in a muscle under scorpion venom. A, 3 per second; B, 5 per second.

When neuromuscular transmission has been blocked by curare, scorpion venom given by intravenous injection decurarizes (2). However, large doses of the venom produce a complete block of neuromuscular transmission. This block is not suppressed by either prostigmin or curare. The muscular responses to direct electrical or acetylcholine stimulation are preserved (Figs. 7 and 8).

Scorpion venom displays anti-cholinesterase activity as shown by our experiments testing the fall of blood pressure produced by fixed amounts of acetylcholine after hydrolysis with blood serum with and without venom.

This property was also assayed comparatively with eserine on frog abdominal muscles (5). The anticholinesterase activity of different venoms was found to be slight and without correlation with toxicity or the muscle activating properties.

A study of the effects of venom (*C. noxius* Hoffmann) on cholinesterases was examined on the isolated intestines of guinea-pigs and rabbits. It was found that the venom only inhibit cholinesterases both from human serum (pseudo) and from the caudate nucleus of rabbits (specific) when present in very high concentrations (6).

In brief, the muscular effects of scorpion poisoning are produced by two actions of the venom: one, central, located on the spinal cord, and the other, peripheral, on the neuromuscular junctions. Both actions take place at regions
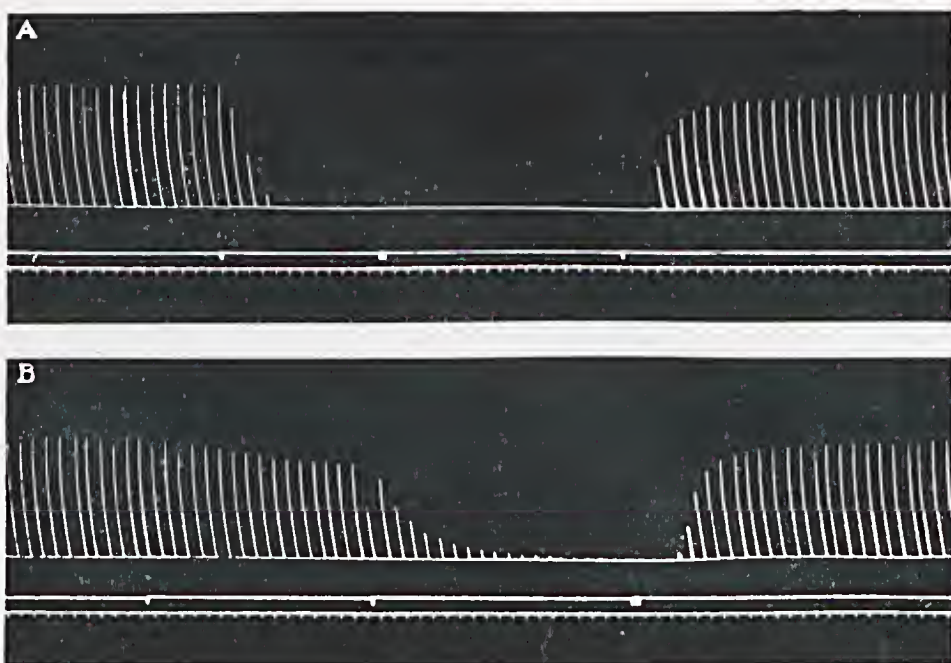
Fig. 7 — Decurarization by scorpion venom (B) and by prostigmine (A). The first signal in both segments correspond to the injection of curare. The second in B to a second injection of curare (in A to atropine). The third signal in B marks the time of the intravenous injection of scorpion venom. (In A, prostigmine).
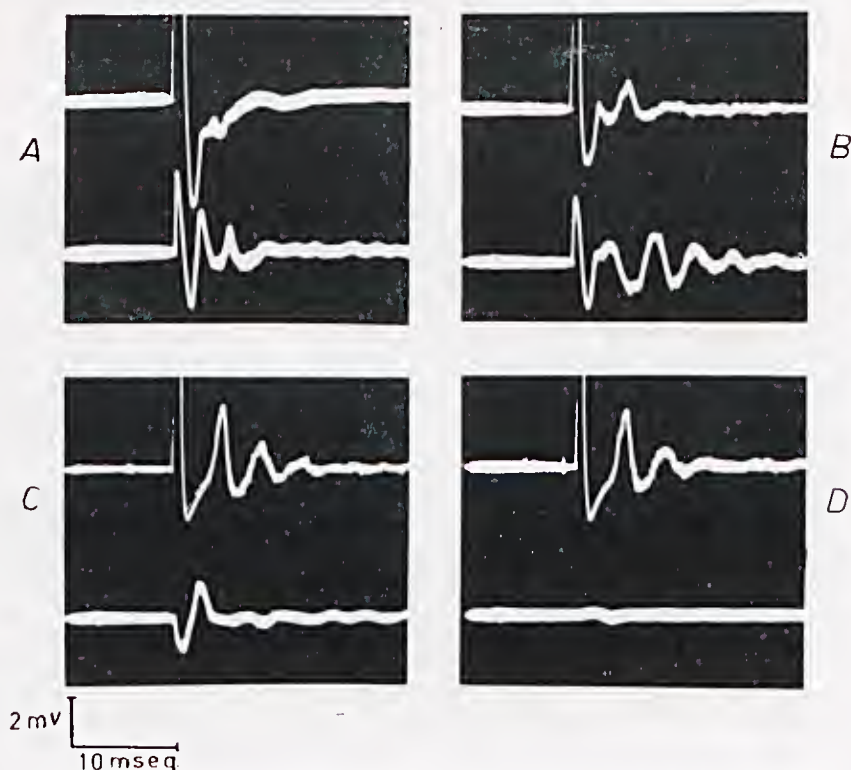
Fig. 8 — Simultaneous recording from sciatic nerve (above) and gastrocnemius muscle (below) to stimulations applied to the nerve. A, before, B, 1 minute after injection of scorpion venom. C, 5 minutes later. D, after curare.

of excitable membranes, and it is reasonable to assume that the venom affects the permeability of these membranes and change the ionic equilibrium between both sides of the same.

Macroelectrode recordings from the end-plate regions of the sartorius muscle of a cat showed a progressive inversion of the local potential under the action of scorpion venom (7). This is a direct evidence of changes in ionic distribution produced by the scorpion toxins.

Respiratory effects — The accidental or experimental poisoning with scorpion venom produce marked irregularity in different animals both in frequency and amplitude of the respiratory movements which in cases of strong intoxication reaches a Cheyne-Stokes type rhythm and finally paralysis. The central origin of this paralysis was attested by keeping the animals (cats) alive after the paralysis by means of artificial respiration brought about by rhythmic stimulation of both phrenic nerves. In these experiments, to our knowledge, was for the first time applied what afterwards was introduced under the name of *electrophrenic respiration.* The paper published in 1945 refuted the hypothesis of curarization as the origin of the respiratory paralysis (8).

Bronchiolar obstructions by abundant secretions, laryngeal and bronchiolar muscles contractions contribute to the respiratory distress but are not the cause of the asphixia. Same may be said of the fascicular contractions that appear in respiratory as well as in all skeletal muscles. Periodic rhythms of respiration from central origin can be perceived among the background of irregular jerks up to the time of appearance of Cheyne-Stokes type rhythms.

An additional proof of the central action is that a minute amount of the venom injected into the *cisterna magna* produces immediate respiratory paralysis.

Cardiovascular effects — The intravenous injection of scorpion venom produce in cats and other mammalians an initial increase of blood pressure and simultaneous bradicardia. The pressure then comes down slowly to normal values. During the periods of cyclic respiration, increases in blood pressure accompany the periods of apnea and blood pressure falls during hyperventilation. Finally, if the dose of venom was high the pressure comes down gradually to zero (9).

The initial increase in blood pressure long time ascribed to a peripheral action of the venom is due to effets on the spinal vaso-constrictor presynaptic neurons and to the liberation of epinephrine also through stimulation of the adrenal activating pre-ganglionic neurons of the spinal cord.

Spinal animals after total destruction of the brain, give equal or higher blood pressure increases with the venom, but the effect does not appear when the spinal cord is destroyed even when the blood pressure level is previously raised to normal values. Denervated pinnae of white rabbits and cats showed vaso-constriction due to liberated epinephrine; it did not occur when the sympathetic fibers to the glands were cut or the spinal cord was destroyed (Figs. 9 and 10).

Bradicardia corresponded also to a central action. It disappeared when the medula was destroyed or when the vagi were cut. In these cases the heart was slightly accelerated under the influence of scorpion venom. This effect was also of central origin because it did not occur when the heart was denervated.

The vascular reflexes produced by the stimulation of the central end of the sciatic nerves peripherally cut were not modified by scorpion venom on curarized
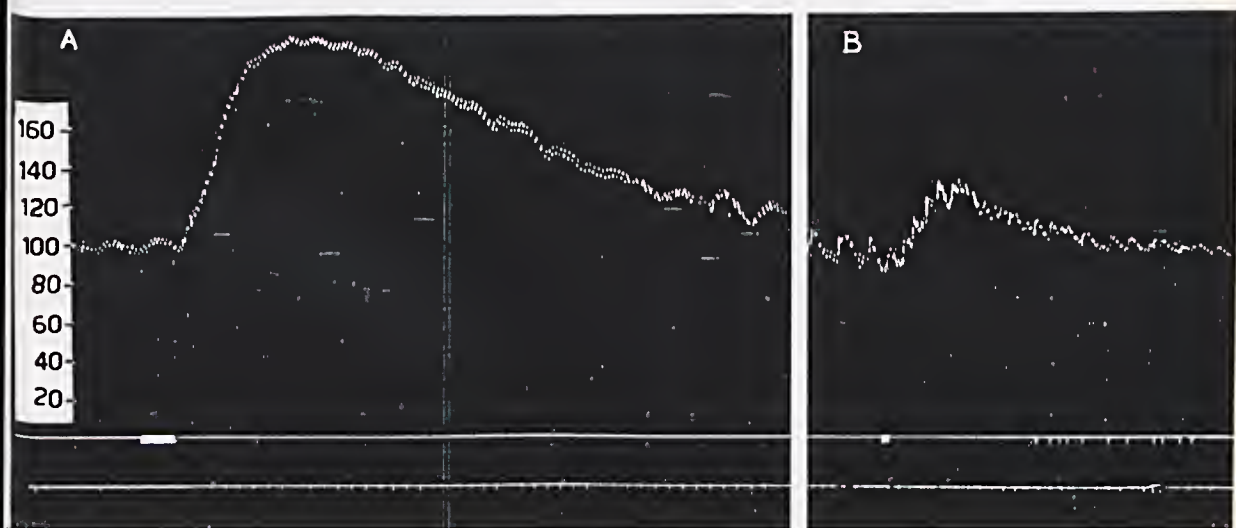
Fig. 9 — Increases of blood pressure produced by a first (A) and a second (B) intravenous injection of scorpion venom in a spinal cat by destruction of the brain. (This and the following figure are taken from del Pozo, Anguiano and González (9)).
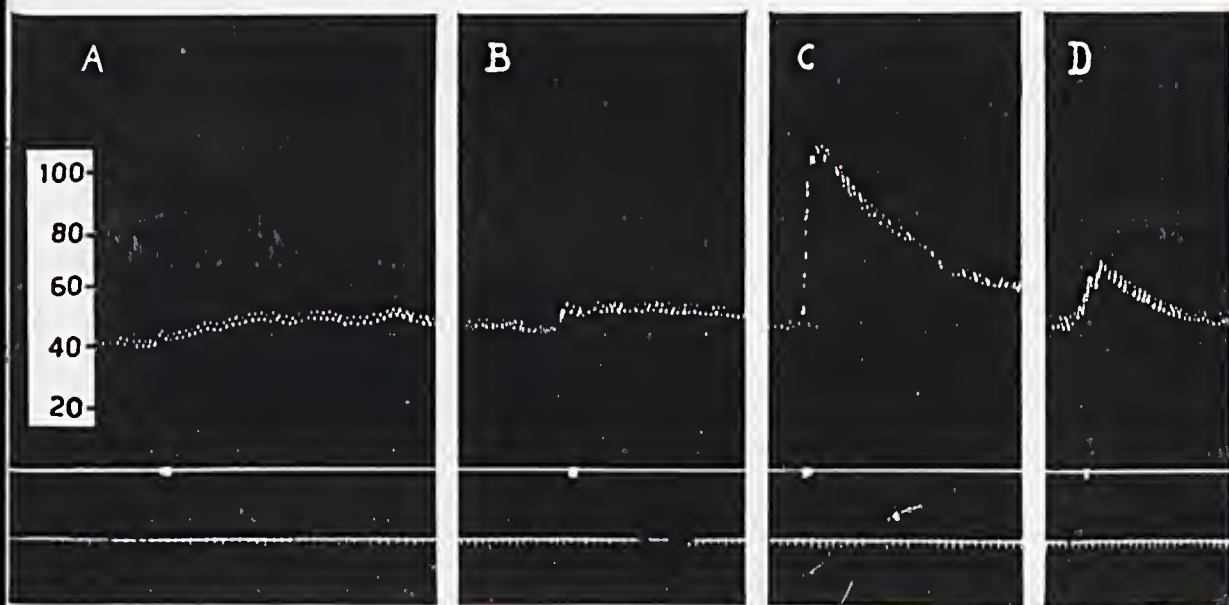
Fig. 10 — Scorpion venom does not produce increase of blood pressure when brain and spinal chord are destroyed. A, first injection of venom; B, injection of an equal volume of saline; C, injection of 10 mcg of epinephrine; D, 5 mcg of epinephrine.

animals. This dissociation between the tonic vasopressor direct effects and the reflex responses is an old physiological finding reported by Sherrington in 1906 (10), Porter in 1910 (11) and Langley in 1924 (12).

OTHER EFFECTS — Other effects of the scorpion venom such as ptyalism, mydriasis, piloerection are also to be explained by central action on the sympathetic pre-ganglionic neurons because those effects are greatly reduced or disappear completely by denervation.

We have seen that the rich pharmacological actions of scorpion venoms seem to derive from a fundamental common mechanism present at different anatomical regions. We have discovered already that this venom acts on the typical places so-called *centers* and at neuromuscular junctions. We know that it works by increasing the permeability of excitable membranes as shown by the inversion of end-plate potential (7). Others investigators have reached similar results with finer techniques (13).

In addition, valuable biochemical work is being done in several laboratories for the isolation of scorpion toxins; we may be hopeful that now we are on the road to a basic understanding of the mechanism of action of these peculiar venoms.

This first International Symposium has brought together the people working on the same problems in all parts of the earth. It will be an historical event. The importance of this meeting is increased because we meet at this famous Butantan Institute, the leader of the venomological studies. We pay tribute to the illustrious Vital Brazil, inspired founder of the Institute and to all the group of workers that have followed his steps in this fascinating path of research.

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Discussion

H. Edery: "All these features you described on the neuromuscular transmission such as twitchings, and fasciculations are also produced by anticholinesterase organophosphorous compounds. I learnt from your paper that you tested the venom for anticholinesterase activity and it has a high concentration. I would like to ask if you would accept the idea that at least part of the effect on neuromuscular transmission were due to inhibition of cholinesterase at the end-plate level. When you inject a substance intraarterially, you do get high level concentrations. And thinking in this way of inhibition of cholinesterase, I would be keen to know the effects of the so-called reactivations of cholinesterase on the block produced by the venom."

E. del Pozo: "We did find anticholinesterase activity and we thought that this could be the explanation for the muscular effects of scorpion venom. However, when tested that possibility we found that the anticholinesterase effect was slight and did not keep relation or correlation with the muscle activating properties. For this last testing we compared venoms from different scorpion species with regards to anticholinesterase activity, toxicity and muscle activating properties. One particular venom could have less anticholinesterase and more muscle activating properties than other."

P. Efrati: "Also my experience concerns mainly stings by *Leiurus quinquestriatus*, I think, at least two clinical signs, observed in general envenomation, could support the observation of E. C. del Pozo about the influence of scorpion venom on the spinal cord: urinary retention and priapism, observed very often indeed."

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64. PHARMACOLOGY OF THE POLYPEPTIDES FROM THE VENOM OF THE SPIDER *PHONEUTRIA FERA*

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This paper resumes pharmaco-biochemical investigations made with the venom of the spider *Phoneutria fera* Perty, 1833 (*Ctenus ferus*, and *Ctenus nigriventer* Keyserling, 1891), a species of the **ARANEIDA** order, *LABIDOGNATA* suborder, CTENIDAE family and CTENINAE subfamily.

Very dangerous by its aggressivity and venom toxicity, this species is responsible for the higher lethality percentage amongst other venomous species from the State of São Paulo. The individual extractions of these spiders may furnish a maximum of 1.8 mg dried venom in winter and 2.5 mg in summer (1, 2). After dried the venom has a grayish-white colour. Notwithstanding its toxicity being comparable to that of *Crotalus* and *Bothrops* ophidian venoms, the small available venom amounts of individual spiders are generally only capable of attaining lethal concentrations in children.

VENOM EFFECTS ON DOGS

In dogs the *P. fera* venom was preferentially administered by subcutaneous route due to its high toxicity and the intense blood pressure drop it induces when injected intravenously.

Subcutaneous low lethal doses (180-200 μg/kg body weight) provoke in dogs successively: intense local pain, violent sneezes, lacrimation, abundant sialorrhea, vomiting, priapism, prostration, semen elimination and in some cases death. Sublethal doses generally are insufficient to induce priapism by subcutaneous route. With the exception of priapism (3), all the aforementioned venom effects were anteriorly reported by Vital Brazil and Vellard (4).

Local pain — The venom subcutaneous injection is excruciatingly painful, it makes dogs yelp for nearly an hour, forcing them to maintain contracted the injected hind leg for longer periods. The pain factor is neutralized by the specific antivenin, which excludes the possibility of this effect being caused by venom contained histamine or serotonin. On the other hand, the pain factor being dialyzable, its molecule must be relatively small to be considered as a bradykinin releasing enzyme. Priapism onset may take place during the pain period.

Sneezes — In dogs sneezes constitute one of the first envenomation signs. They are intermittent being observed for more than 24 h. The first attacks are very strong, the animal throws its head violently and uncontrollably towards the floor, very often, hurting its nose and lips.

Lacrimation and mydriasis — During envenomation tears drop constantly from the dog eyes, even in anesthetized animals.

Mydriasis is induced early after venom injection, it persists for many hours. The dogs present visual disturbances as a consequence of mydriasis.

Sialorrhea — The venom provoked sialorrhea is abundant, pilocarpine-like. Unswallowed salive drops from the animal mouth continuously for hours. This effect is blocked by atropine and doses of eserine and hexamethonium, which increase to a small extent the poisoning signs, seem not to interfere in its manifestation (3). Subcutaneous effective doses are ineffective in dogs under chloralose, chloroform and barbiturics anesthesia, however, the same doses induce sialorrhea when applied intravenously.

Priapism — The venom induced priapism is also an intermittent and long-standing effect (Fig. 1). It manifests repeatedly for hours, very often exceeding 24 h, in which cases edema is generally formed at the penis distal extremity correspondent to the glans.

Fig. 1 — Priapism induced by *P. fera* venom. The dog presents: adynamia, locomotion difficulties and drowsiness.

Priapism generally takes place at a more advanced envenomation phase when the dog has already presented intoxication signs. Differing from sialorrhea, venom priapism could not be induced in anesthetized dogs even when the venom was administered intravenously in larger doses than those which are effective by subcutaneous route. Priapism is induced in dogs having the medulla cut at DXII.

indicating that it is independent from the excitation of higher centers (5). Unlike cantharidin priapism, this of the arachnidan venom does not seem to result from reflexes caused by irritation of the urinary tracts. In dogs, the venom provokes priapism before any micturition, as well as in animals with both ureters implanted into the skin; it is also not evoked by perfusing venom solutions through the urethra into the bladder.

Semen elimination — In dogs the *P. fera* venom provokes elimination of semen during or after priapism onset. This effect seems to result from the excitation of other structures than the seminal vesicles since dogs have not got them (5).

Toxicity — The venom intoxication pattern of dogs is characterized by: adynamia, locomotion difficulties, prostration, drowsiness, vomiting, dyspnea, sanguinolent feces and death. By using 200 μg/kg subcutaneously, the dog survives for several hours before death occurs. Dogs also present sneezes, sialorrhea and priapism during the severe intoxication phase.

Guinea-pig ileum contraction — C. Diniz separated from this venom two polypeptides which contract the ileum of guinea-pigs; according to his findings the fraction containing one of these polypeptides was also responsible for the venom toxicity (6).

Blood pressure fall — Endovenously, small venom doses provoke a sharp blood pressure fall. The too small histamine content of these venom doses have no comparable effects on blood pressure.

Tachyphylaxis — Rafael R. L. Sampayo (7) reported that components of the black widow spider (*Latrodectus mactans*) venom provoke tachyphylaxis in dogs. Tachyphylaxis phenomena were not observed for any one of the effects elicited by the *P. fera* venom. The intermittent actions would not have this characteristic in the case that the agents responsibles for these effects would have tachyphylaxis properties. Tachyphylaxis also shows that both these venoms differ by the molecular structure of their components.

Venom effects on mice

C. Diniz was able to reproduce on albino mice the *P. fera* venom priapism first observed in dogs (8). Excluding lacrimation, mydriasis, sneezes, semen elimination and vomiting, which are difficult to be followed in mice, all the other *P. fera* venom effects can easily be tested on these small rodents (5).

Mice strain and weight are essential for reproductive quantitative assays. The best responses, for mice of the Instituto Butantan strain, are obtained by using animals of 22-25 gm. With less than 20 gm they are more sensible to toxic effects than to priapism. The method of Reed and Muench (9) shown to be very suitable to estimate statistically this venom actions on mice.

Local pain — Not so noticiable as in dogs, it is, however, exteriorized in mice by contraction of the injected hind leg. The animals also bite the injection area in a scratching form.

Sialorrhea — As Fig. 2 shows, this effect can easily be followed in mice. At the beginning small bubbles of saliva accumulate at the mouth. Later on, depending on the venom dosage, the animals may have a large part or nearly all of its fur wet with saliva. Small doses of venom provoke sialorrhea without any sign of toxicity. With larger doses, sialorrhea appears before toxic manifestations and continues on its appearance. The venom ED50 for sialorrhea is 0.43 mg per kg body weight.

Fig. 2 — Beginning of *P. fera* venom provoked sialorrhea in mice. The mouse is apparently normal, no sign of intoxication is evident.

Priapism — Fig 3 shows clear manifestation of priapism in mice. The priapism dose-effect relationship in mice is maintained until a maximum of responses in a group of animals are attained, thereafter, increasing doses of venom provoke a decrease in the number of animals which present priapism, and death occurs in some of them before this effect appears. In a similar way to sialorrhea, small doses of venom can induce priapism in mice without any toxic manifestations; in this point mice react differently from dogs. The priapism ED50 for mice is 0.25 mg per kg body weight.

Toxicity — The venom toxicity manifestations in mice are: dyspnea, prostration, distensive paralysis and death. The toxicity LD50 is 0.76 mg per kg body weight.

Distensive paralysis — As can be seen in Fig. 4, in mice crude venom provokes a distensive paralysis of the hind legs and tail; this latter, during a certain period, remains bent in a distensive form upon the animal back. The

Fig. 3 — Priapism induced in mouse by 10 μg of *P. fera* venom. This dose is effective for priapism and sialorrhea but does not provoke intoxication signs.

Fig. 4 — *P. fera* venom distensive paralysis. Characteristic position of hind legs and tail; the mouse died in a precocious rigor mortis.

hind legs paralysis takes place after the induction of sialorrhea and priapism and remains until death, which occurs with the animals already presenting a precocious sort of rigor mortis.

Venom effects on other rodents

Guinea-pigs reproduce almost all the venom actions provoked on dogs and mice, however, they have been less investigated than mice due to their larger venom consumption (3, 5).

Rats and rabbits are very resistant to this venom actions, 500 μg on rats of 150 gm only provoke secretion of the glands of Harder, and 1,000 μg on rabbits of 2,000 gm only induce small sialorrhea and light intoxication signs (3).

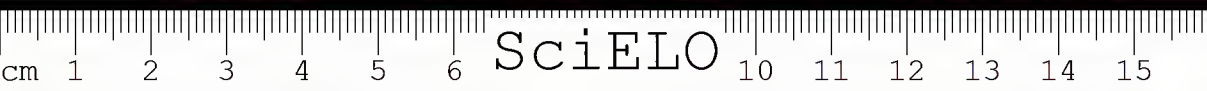
Human being accidents

A pattern which resembles that of dog envenomation is also noticed in human beings suffering from *P. fera* stings: local unbearable pain, sialorrhea, visual disturbances, sweating, prostration, priapism and death. Priapism is predominantly observed in boys under 10 years presenting signs of severe intoxication. Generally patients do not complain about priapism, and it remains unperceived by the clinicians. In the last years, aware of this venom effect, clinicians have given more attention to this symptom of *P. fera* stings, principally in the cases brought to the Instituto Butantan Hospital.

Biochemistry of the *Phoneutria fera* venom

Some publications have been made regarding the constituents of this venom. A. Barrio reported neuromuscular and muscular actions of its electrophoretic fractions (10). C. Diniz demonstrated by electrophoresis and chromatography that it contains histamine, serotonin and two polypeptides which contract the ileum of guinea-pigs (6). F. G. Fischer separated from it histamine and serotonin by electrophoresis, demonstrating that this venom also contains, in free form, glutamic acid (23.6%), aspartic acid (1.0%) and lysine (0.2%) (11). J. H. Welsh studied comparatively its serotonin (12). An hyaluronidase and a proteolytic enzyme of this venom were reported by E. Kaiser (13). The histamine content of the *P. fera* venom varies, according to different reports, from 0.06% to 1.0% and this of serotonin from 0.03 to 0.25%.

Antigenicity — Excluding the venom small molecules (histamine, serotonin), all the others active constituents are neutralized by the specific antivenin, a fact which attributes to them characteristics of large molecules of a probable proteic nature (14).

The venom antigenic composition, determined by agar double-diffusion immunoprecipitation (Ouchterlony method) and also by agar immunoelectrophoresis, showed that it must contain approximately 14 antigens (15). These findings pointed out the possibility that the venom might have several distinct components, each one corresponding to one or more of its effects. They also permitted foreseeing the separation possibilities of the toxic from the non toxic factors, and

these from others probably not yet identified active venom principles that, once attained, would provide better conditions for proceeding with the investigation of their pharmacological properties.

Flaccid paralysis effect — In the course of these immunological determinations, a venom fraction was separated by electrophoresis in agar plates, pH 5.0, which induced flaccid paralysis in mice when injected subcutaneously (Fig. 5).

Fig. 5 — Flaccid paralysis induced in mice by Sephadex G-50 fraction of *P. fera* venom heated at 100°C for 6 min. in phosphate buffer, pH 8.0, 0.05 M. The hind legs are paralysed in a flaccid form; one of the mice is trying locomotion by using the fore legs; both presented flaccid bodies after death.

The same component was also separated later on in cellulose acetate strips, pH 5.0 (16). This paralysis differs from the distensive one, the hind legs are paralysed in a flaccid but not distensive form, as that which is provoked by crude venom. The tail also remains flaccid, and the animal motionless; for its locomotion the fore legs are used to pull the body. This fraction is very toxic, and death occurs with the mice presenting a flaccid body, also differing, in this particular, from the crude venom lethality in which the animals die in rigor mortis. Fractions containing the principle responsible for this effect were also separated by other methods and will be described later on. In crude venom assays the distensive paralysis dominant effects act as functional antagonizers of the flaccid paralysis actions, which could only be revealed after separation of both their components.

Heat inactivation — All the venom pharmacologically active components are resistant to heating. Venom solutions in saline of mammals can be heated at 100°C for 20 min without losing activity. However, inactivation occurs when

heated at the same temperature for 6 min at pH 8.0; the recovered activity of some of the components being of: ileum contracting polypeptides — 100%, priapism — 39.2%, sialorrhea — 34.2% and toxicity — 19.1% (17).

Chemical and enzymic inactivation — The venom effects are inactivated by concentrated acetic acid. The priapism, sialorrhea, toxicity and ileum contracting effects are more resistant to acids (HCl 0.1 N) than to alkaline solutions (NaOH 0.1 N). The components corresponding to these four effects are inactivated by NaOH 1.0 N.

Sulphuric ether, acetone, chloroform and butanol do not inactivate any of these effects, the first three extract from the venom a significative amount of an inactive material together with some of its histamine and serotonin (15).

Excepting the venom histamine and serotonin effects, all the others are inactivated by trypsin, chymotrypsin and pepsin, indicating the proteic nature of their corresponding components (15, 17).

The proteolytic enzyme of *P. fera* venom inactivates all its own large molecule constituents, however, much more rapidly the guinea-pig ileum contracting polypeptides than the components responsible for the remaining effects. This enzyme also degrades casein much more rapidly than albumin.

Dialysis — The Visking tube 18/32 (impermeable to insulin) is more permeable to the priapism than to the sialorrhea and toxic principles. All venom active constituents dialyse rapidly through tube 8/32 as also does insulin (14, 15, 17). As these venom components dialyse through tube 18/32 (impermeable to insulin), it must be admitted that their molecular weight is smaller than 5,733 (insulin). So, they must be considered as polypeptides since they are also degraded by the proteolytic enzymes, besides being also immunogenic.

Venom fractionation

Ammonium sulphate and electrophoresis — The flaccid paralysis and the ileum contracting components were separated by 65-75% ammonium sulphate saturation. Electrophorectically, all active components are positively charged and migrate to the cathode. The flaccid paralysis component was the only one separated by electrophoresis (agar plates or cellulose acetate strips) in acetic acid ammonium acetate buffer, pH 5.0 (16, 17).

Gel filtration columns — All the large molecule components are excluded in Sephadex G-25, excepting the ileum contracting polypeptides which have not yet been assayed, indicating that their molecular weights are greater than 5,000, but smaller than 10,000 since they diffuse in Sephadex G-50, data which confirm others anteriorly obtained by dialysis (14, 15, 17). Both seem to indicate that the larger pharmacologically active molecules of the *P. fera* venom have molecular weight larger than 5,000 and smaller than 5.733 (insulin). These components being immunogenic and hydrolyzed by proteolytic enzymes, and having molecular weights between 5,000 and 6,000, must really be considered as polypeptides.

Fig. 6 shows a chromatogram of 20 mg *P. fera* venom run in a Sephadex G-50 column (760 × 22 mm), eluted with phosphate buffer, pH 7.5, 0.05 M and NaCl 0.1 M (15). The venom was previously heated at 100°C for 6 min

at phosphate buffer, pH 8.0, 0.05 M. This treatment denatures the proteolytic enzyme and a large part of the component responsible for the distensive paralysis, permitting to separate the flaccid paralysis component.

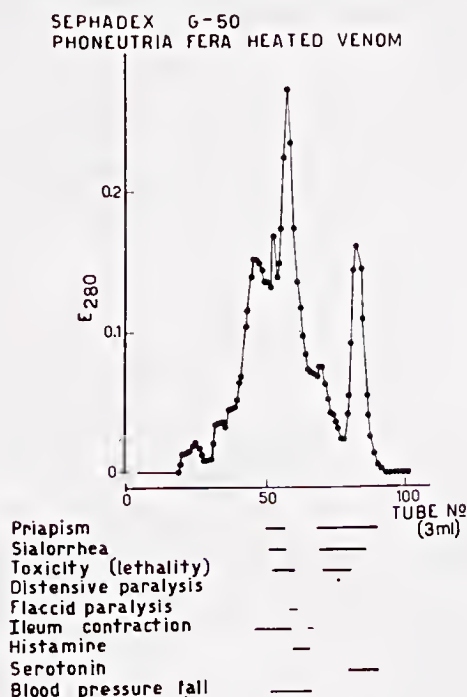



Fig. 6 — Chromatogram of a 20 μg *P. fera* venom run on a Sephadex G-50 (fine) column (760×22 mm). The venom was previously heated at 100ºC for 6 min in phosphate buffer, pH 8.0, 0.05 M. 13 pharmacologically active components, distributed in two zones, are represented; flaccid paralysis was revealed after venom heating at pH 8.0.

All the identified effects are found amongst the effluent fractions. The activities are distributed in two zones; they have no relationship with the 280 mμ absorption peaks, which seems to indicate that the venom active polypeptides are poor or deprived of tyrosine, tryptophan and phenylalanine. Some of the same effects are found in the two activity zones, whose components are well differentiated by their molecular size, showing that the venom contains components of different molecular weights having, however, the same pharmacological activity.

The ileum polypeptides, histamine and serotonin were obtained individually in some tubes free from other components, evidencing that probably toxicity and ileum contraction are exerted by different venom molecules. Priapism, sialorrhea and toxicity polypeptides are not separated by Sephadex G-50; tubes which contain the two former probably also contain that of toxicity.

The flaccid paralysis component, by being also toxic, its fraction overlaps a small part of the toxicity line. The venom seems to contain a third toxic component deprived of any paralysing effect.

Ionic columns — Fig. 7 shows the fractionation chromatogram of 20 mg venom in a CM-Sephadex C-50 column (270 × 10 mm), in gradient and step-

wise elutions. Similarly to other columns, overlapping of the effluent activities with 280 mμ lecture peaks does not exist. Four activity zones were formed by this venom fractionation, the same number of zones were also obtained with DEAE-cellulose columns. Histamine and serotonin were not assayed in these fractions, two of the four fractions which contract the ileum must correspond to these amines. Flaccid paralysis, which is separated from distensive paralysis in DEAE-cellulose columns, was not separated by this column, and was not revealed. Toxicity is not represented in the graph under priapism and sialorrhea activities of the first and third activity zones, which would indicate that toxicity, priapism and sialorrhea are not provoked by a single component. However, these findings were not considered as definitive proofs, since large volumes were necessary to induce priapism and sialorrhea, both requiring lower minimal effective doses than toxicity.

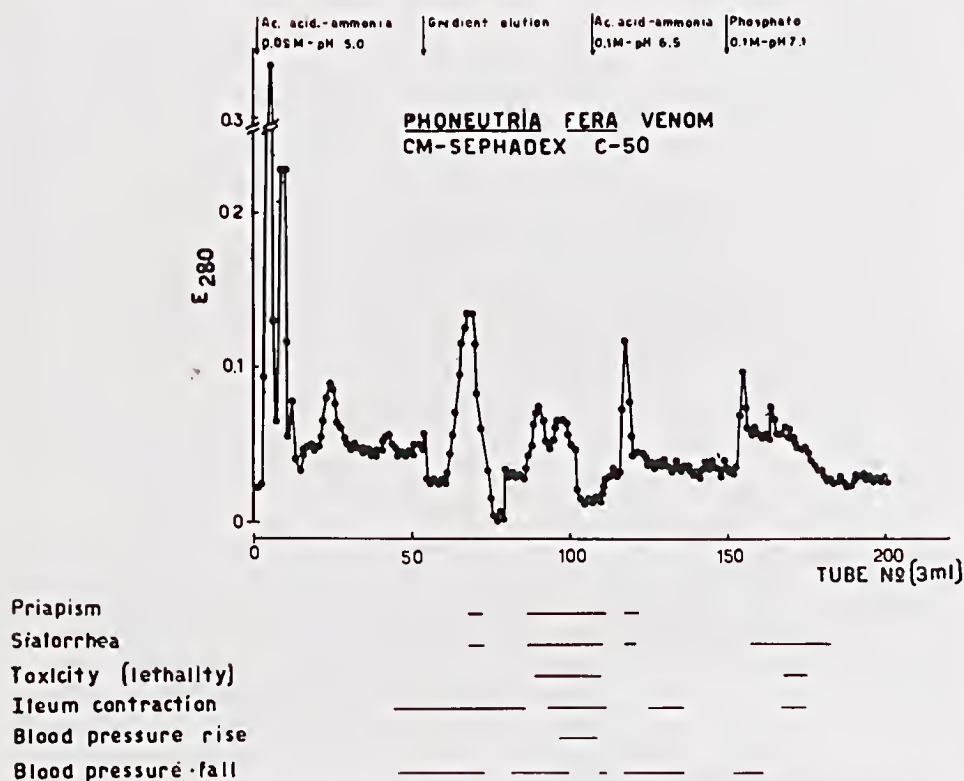



Fig. 7 — Chromatogram of a 20 mg *P. fera* venom run on a CM-Sephadex C-50 column (270×10 mm), in gradient and stepwise elutions. Some of the same effects are distributed in four zones. The priapism effect is absent in the fourth zone, indicating the possibility that its molecules might differ from those responsibles for sialorrhea and toxicity.

Very significant, however, is the absence of priapism in the fourth zone, since its minimal effective dose is smaller than that of toxicity, which was induced without any priapism manifestation, demonstrating the possibility that priapism and toxicity might be provoked by two distinct polypeptides.

Two other components were separated by this and also by Sephadex G-50 columns. Administered by intravenous route, one provoked blood pressure rise and the other blood pressure fall. Both have not been well studied, nevertheless, some of their fractions are not contaminated with histamine or serotonin. In crude venom the hypotensive effects dominate and mask the hypertensive ones (17).

These column experimental findings furnish evidences which seem to demonstrate that the molecules of each activity zone differ from those of other zones in what regards their size and electrical charges. Those of each zone also differ one from each other by their pharmacological properties. According to these data, the venom would contain 16 pharmacologically active components, including histamine and serotonin.

The problem increases in complexity when it is admitted that each one of some of the venom effects can be provoked by four different polypeptide molecules. It would be difficult to admit that the veneniferous glands would secrete such a number of components provoking, some of them, the same effects, that is to say, having the same finalities.

A better investigation of the venom proteolytic enzyme might throw some light on these apparently paradoxical facts. This enzyme differs from trypsin, chymotrypsin and pepsin by its substrate linkage specificity. It seems very probable that the veneniferous gland would secrete the proteolytic enzyme together with a few large molecules, having one or more of these particular linkages. Depending on the stage of these large molecule cascade degradation by the venom proteolytic enzyme, the venom accumulated in the veneniferous gland might present molecules having the same pharmacological active centers bound to larger or smaller chains, and these would condition their differences in size and electrical charge. The degradation process of these large hypothetical molecules should be interrupted at a certain equilibrium point, otherwise all the venom would be transformed into amino acids.

The large amount of free amino acids of the venom seems to represent residues of this degradation, it also constitutes an argument in favour of the above mentioned hypothesis. The different amounts of each one of the three amino acids furnish a light indication of the molecular structure of these hypothetical large molecules secreted by the veneniferous gland. The venom content in glutamic acid (23.6%) seems to show the enzyme preference for a linkage containing this amino acid, presumably it would constitute the terminal amino acid of one or more of the venom molecules.

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Discussion

C. Y. Lee: "Is the flaccid paralysis caused by the fraction of the spider venom central or peripheral in origin? Have you studied its effect on the neuromuscular transmission?"

S. Schenberg: "Studies were not yet made to determine the local of action of the flaccid paralysis component. Work is being done to separate and purify this polypeptide as to permit a better investigation of its pharmacology."

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65. SPIDER GLANDS AND PSYCHOTROPICS

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There exists evidence that the silk glands of the spider *Araneus diadematus* Cl. work in close connection with the central nervous system (CNS). The CNS appears to send signals to the glands, "telling" them to produce more or less silk; it seems also "informed" of the amount of silk available in the ampullate glands at a given time. Silk being so important in the spider's life — for trap-building, moving around, and communication — such two-way mechanisms appears of great survival value: enough material is made available, but no energy is wasted on excess. The close interrelationship between CNS and glands explains that drugs which affect CNS function, the psychotropics, influence silk gland activity and that webs built after these drugs reflect their effects in web-weight and -pattern. The evidence will be reviewed.

To establish the amount of silk which a spider uses for one web, the whole structure is cut off from its supports, rolled up on a piece of relatively nitrogen-free filter paper, and digested in selenium-sulfuric acid. The amount of nitrogen determined with an optical micromethod in the web digest is a measure of the total amount of polypeptide in the web. Fig. 1 shows the results of an experiment with 23 spiders which were treated with 1 mg/kg physostigmine by mouth in sugar water and 23 control animals. The webs were digested daily and on Tuesday both groups showed the same mean of 39 microgram N per web. 36 hours after the drug had been given, on Wednesday morning, the physostigmine-treated animals had built webs containing a mean of 48.9 ± 4.4 microgram N per web, while controls built significantly lighter webs with 20-30 micrograms N. The following day, Friday, all animals, controls and drugged spiders, built webs with the same mean of 34.5 microgram N per web. It was concluded that webs after physostigmine are heavier or contain more polypeptide thread.

In order to test the hypothesis that the cholinergic drug stimulates silk production rather than promotes a more thorough squeezing out of the ampullate silk glands, Peakall performed experiments using 3 kinds of methods (1, 2):

A: Glands were pulled empty of thread at regular intervals, f.e. every 6 hours, and the quantity thread pulled was determined in the selenium-sulfuric acid digest. Such procedure takes advantage of the experience that a spider which sits on a rough surface lets out thread onto the rotating axle of a motor until the glands are empty. Peakall's results with physostigmine and carbachol, 2 cholinergic drugs, and atropine, an anticholinergic drug, show that with increasing dose of the drug, silk quantity increased after the cholinergic and decreased after the anticholinergic drug.
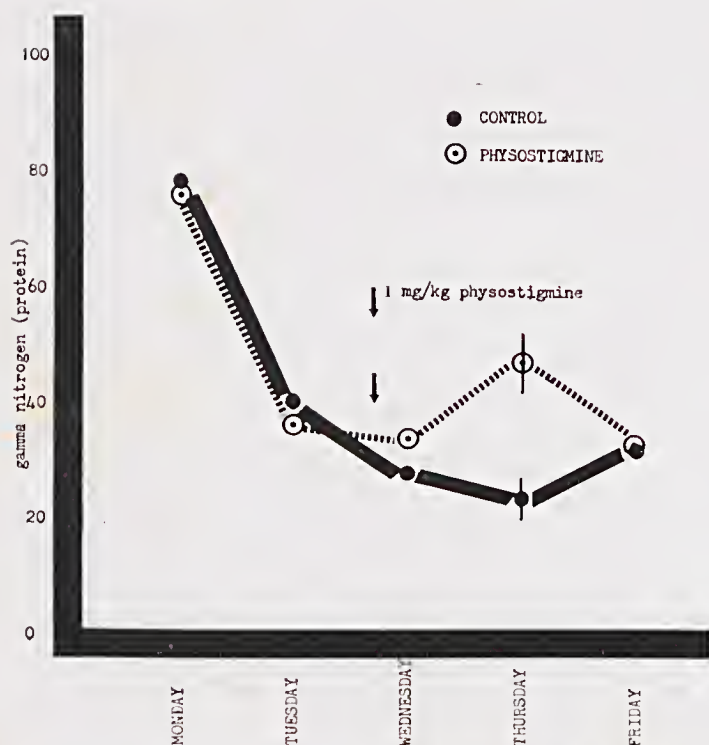
Fig. 1 — Nitrogen values in the digest of webs of 23 spiders treated with 1 mg/kg physostigmine on Tuesday night (12 hours before the Wednesday web-building time) and 23 controls. Each point represents the mean of all measurements, and the vertical line the standard error of the mean. The two Thursday values are significantly different below the 1% probability level. Note the increase of web nitrogen 36 hours after the drug.

B: Another procedure uses labelling of a silk precursor, alanine, with C14, injecting it into the spiders' abdomen, and measuring the speed with which the label turns up in the silk. 6 hours after alanine was given, significantly more label appeared in the silk after physostigmine, carbachol and paraoxon than in untreated spiders (Table I). It was also found that just emptying the glands promoted incorporation of the label, and that emptying plus drug treatment was not additive, but rather made the glands behave like after one treatment alone. We can therefore assume that two mechanisms regulate speed of silk production: 1) a feed-back from the lumen of the gland via the inner epithelial membrane and 2) a possibly neurohumoral mechanism via the outer epithelial membrane.

C: A third way of measuring changes in silk production is the use of radio-autography: the labelled silk precursor, again C14 alanine, leaves black dots on a photographic film which is spread thinly over the sliced tissue. By using this method at different times after alanine injection, its progress from the body fluids into the gland epithelium and from there into the gland lumen can be followed. Table II shows that 4 hours after injection of C14 alanine, most of the label had left the intestine and blood of the spiders and appeared in the gland lumen in pulled or physostigmine treated animals, while atropine treated

TABLE I — AMOUNT OF INCORPORATION OF C-14-ALANINE INTO WEB PROTEIN DURING 6 hr.

The labelled alanine was given at the start of the experiment. Thread was pulled for determination 6 hr afterwards. (From D. B. PEAKALL, *Comp. Biochem. Physiol.*, **12**, 467, 1964).

TREATMENT	No. of spiders	Activity counts/min	Standard error of the mean	Micrograms nitrogen/ 100 mg b.wt.	Standard error of the mean
Unpulled, no drug	15	123	11.1	—	—
Unpulled, physostigmine	18	290	27.4	—	—
Unpulled, carbachol	14	301	25.4	—	—
Unpulled, paraoxon	6	329	60.7	—	—
Pulled, no drug	18	314	13.5	15.0	1.4
Pulled, physostigmine	20	346	17.9	16.2	1.6

TABLE II — COUNTS FROM 7-DAY AUTORADIOGRAPHS OF INTESTINE, BLOOD AND SILK GLAND EPITHELIUM AND LUMEN IN THE INTACT SPIDER

Counts are per mm^2 at magnification of 60. Each figure represents the average, with standard deviation, from 50 counts (10 counts from each of five spiders). Background count (Bkd) were 1-4/mm^2. (From D. B. PEAKALL, *Comp. Biochem. Physiol.*, **15**, 511, 1965).

Time in hr	Treatment	Hind intestine	Blood	Epithelium of silk gland	Lumen of silk gland
2	None	61.0 ± 8.0	29.7 ± 4.3	4.4 ± 1.9	Bkd
	Re-pulled	55.1 ± 6.9	26.3 ± 5.1	8.8 ± 3.7	Bkd
	Physostigmine	54.9 ± 7.9	29.0 ± 2.9	6.1 ± 1.9	Bkd
	Atropine	64.1 ± 9.1	27.1 ± 5.0	Bkd	Bkd
4	None	15.1 ± 2.5	17.8 ± 3.0	25.5 ± 4.1	4.9 ± 0.8
	Re-pulled	10.3 ± 1.8	13.0 ± 5.2	54.3 ± 8.3	18.0 ± 4.2
	Physostigmine	8.4 ± 2.1	8.9 ± 3.1	48.0 ± 7.5	12.5 ± 4.3
	Atropine	16.7 ± 3.0	22.1 ± 4.6	25.5 ± 4.8	6.1 ± 2.1
8	None	Bkd	31.8 ± 1.9	33.8 ± 4.8	38.4 ± 4.0
	Re-pulled	Bkd	4.7 ± 1.9	15.3 ± 2.8	55.6 ± 4.2
	Physostigmine	Bkd	5.6 ± 2.7	16.2 ± 3.5	62.8 ± 9.8
	Atropine	Bkd	14.0 ± 7.4	44.4 ± 9.6	22.4 ± 7.5

or resting glands had not yet taken up the bulk of the label. In histological slides the size and shapes of the ampullate glands and the position of the label can be identified.

If we assume that acetylcholine is the neurotransmitter substance which is responsible for carrying the signal for silk production from the nerve to the gland tissue, we must look for places on the gland which could bind acetylcholine. Peakall's work shows autoradiographic proof that labelled acetylcholine is accumulated on the gland epithelium. This is possibly the area for reception of the neurohumoral signal.

Let us now take a look at the whole animal and see the geographical location and possible interrelationships of silk glands and CNS in *Araneus diadematus* Cl.

F. Meier (personal communication) has identified nerves leading from the big subesophageal ganglion in the cephalothorax to the silk glands. Such nerves could carry signals in both directions, coordinating leg movements of the web-building spider with silk supply in the gland.

Behavioral experiments with web-building spiders have shown that there are 3 possible ways which can lead to webs built with a shorter thread:

1. Atropine sulfate, 1, 2 or 4 mg/kg, was given by mouth to 19, 19 or 39 spiders 12 hours before web-building time. The two lower doses caused webs which showed no change in size or regularity but were built with wider meshes, covering the same area with less thread. The highest dose caused significantly smaller and less regular webs built with less silk. This latter change lasted through the second day after drug application. The interpretation assumes that atropine shows its effect on polypeptide synthesis in the spider's glands as well as interferes with centrally regulated exactness of movements. It is interesting to note that not the size of the catching area in the web was decreased when less thread was available, but the shorter thread was wider spaced so that the trap was full size and only lost the smallest insects.

2. Spiders also built webs with shorter thread after a weight had been attached to their backs. These experiments (3) were undertaken to test the hypothesis that psilocybin, the hallucinatory mushroom poison, caused in spiders similar effects as an increase in body weight. This substance is known to change in man perception of one's body. Does a spider after psilocybin "feel" heavier and therefore builds a weight-web? It could be shown that 150 mg/kg psilocybin given to 9 and 23 spiders in two independent experiments 12 hours before web-building time, as well as a 30% increase in body weight of 15 spiders, decreased average thread length significantly by about 30%. However, when the webs were digested and N determined, a significant difference in the amount of silk was found between the psilocybin and weight-webs: the webs after psilocybin were built with less silk, the shorter thread was as thin as before; the heavier spiders, in contrast, built webs with thicker thread, using equal amounts or even more protein than before. The interpretation for the results of the experiment with heavier spiders assumes that they built a thicker thread to hold themselves up; as they had no more material than usual in their glands, the thicker thread had to be shorter. The psilocybin webs must be interpreted in a different way, as will be seen later. If the interpretation is correct, the spiders' CNS must integrate information on thread length as well as silk quantity stored in the glands during web construction.

3. Experiments performed under the influence of the tranquilizer Valium (diazepam) may help to interpret psilocybin effects (4). When 100 mg/kg Valium were given to 40 spiders 36 hours before web-building time, all animals built smaller webs with a shorter thread and less material. This could be the result of a decrease in silk production in the glands through the tranquilizer, or the glands were not completely emptied at the end of web construction. Experiments with thread pulling after Valium answered the question in favour of the second interpretation: when silk was pulled from 13 spiders one and two days after 100 mg/kg Valium and from 9 control spiders, no difference in quantity could be found. The effect of Valium is therefore interpreted as possibly affecting the spiders' "drive" so that they build smaller webs using only part of the material. The glands stay partly filled at the end of the construction period. This, in time, would slow down new silk synthesis by the feed-back mechanism which was shown by Peakall.

Thus, the three ways in which webs with shorter thread were produced through drugs and weight changes, provide some evidence for the close interconnection between silk glands and CNS. If the tranquilized CNS "instructs" the legs to build a smaller web, less silk is pulled from the glands. Fig. 2

Fig. 2 — *Araneus diadematus* Cl. sitting on a rough surface and facing away from the camera. Note the posterior spinnerets from which the 8th leg pulls two threads, and the anterior spinnerets from which a thread runs to the ground.

illustrates how the spider pulls silk from the spinnerets by means of its hind leg. It can also lower itself on the thread through its weight and regulates speed and possibly thickness with the eighth leg. The funcition of Wilson's control valve (5,6) in this process and its interrelationship with CNS and glands is a matter for future investigations which will we hope further clarify psychotropic drug effects on web-building.

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66. SPIDER WEBS AND PSYCHOTROPICS

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Perhaps, from the viewpoint of the student of venoms, the spider web is nothing more than an elaborate device for delivery of venom to the prey. Granting that interpretation, those of us who are interested in understanding animal behavior are grateful for the intimacy required for the injection of venom: in the orb-web we have a concrete segment of behavior especially susceptible to quantitative study. The web is also a testimony to the sensory and motor integrity of the spider. While it may be further agreed that it is the result of innate behavior patterns, we are not thereby relieved of the requirement to explain the spider's response to contingencies of the moment (e.g. wind and broken strands), nor ultimately to explain the elaborate sensory, nervous and motor systems which mediate those innate patterns.

For it is the case that the technology of web-building is rather imperfectly known. There have been excellent naturalistic observations of the process and several attempts at motion pictures. There have also been experimental interferences in building: by selectively injuring the animal (for instance by removal of legs and by the production of lesions in the central nervous system), by disrupting the ordinary building circumstances (as by destroying threads or rotating the frame during construction), and by feeding the spiders drugs which affect the behavior of many animals, the psychotropic drugs, and which in the spider have reproducible and particular consequences for certain features of the web.

Drug effects on webs have been investigated extensively for two species. *Zygiella x-notata* and *Araneus diadematus*. In these studies, the object of measurement has been the completed web rather than the motions of the animal in the course or act of building. There are many good reasons for this choice, among others the reproducibility of events which occur at great speed under conditions of limited visibility, but a prime consideration has been the variability in the dimensions of the web and the consequent necessity to employ statistical comparisons of many webs, either of many animals under experimental and control conditions or of the same animal treated after a suitably lengthy web-building history had been accumulated.

Fig. 1, which shows the completed web of *Araneus diadematus*, makes it apparent that several different measures or aspects of the web may be chosen for evidences of influence of treatments. The computer, allowing as it does, many and rapid computations to be made, diminished somewhat the limitations of the choice of parameters. It also permitted us to pick up rather subtle changes, apparent in a series of webs but perhaps not in the comparison of two single representatives of treatment and control samples. (See for example Fig. 2, showing a representative drug effect.)

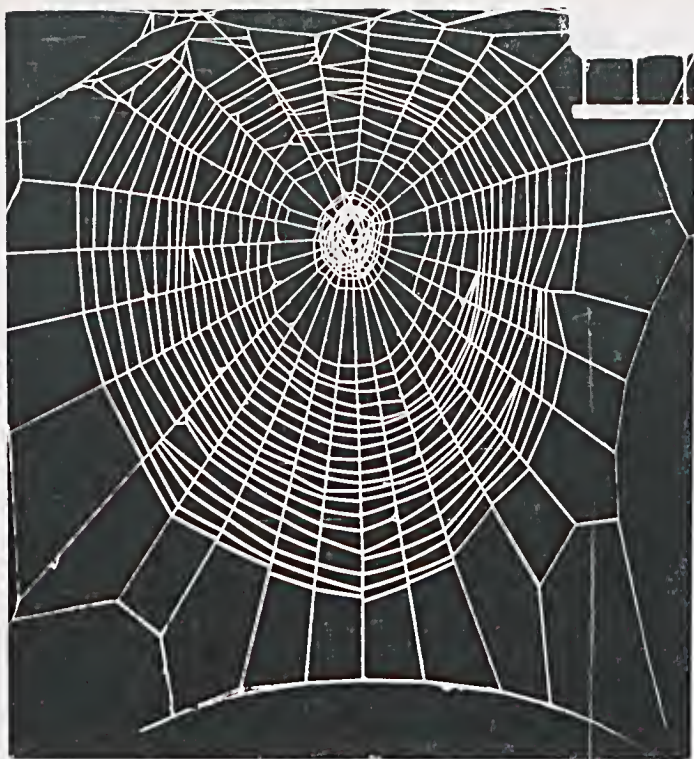



Fig. 1 — Completed web of *Araneus diadematus* Cl. Strueture in upper right eorner is weight supported at 20 mm intervals by three threads.

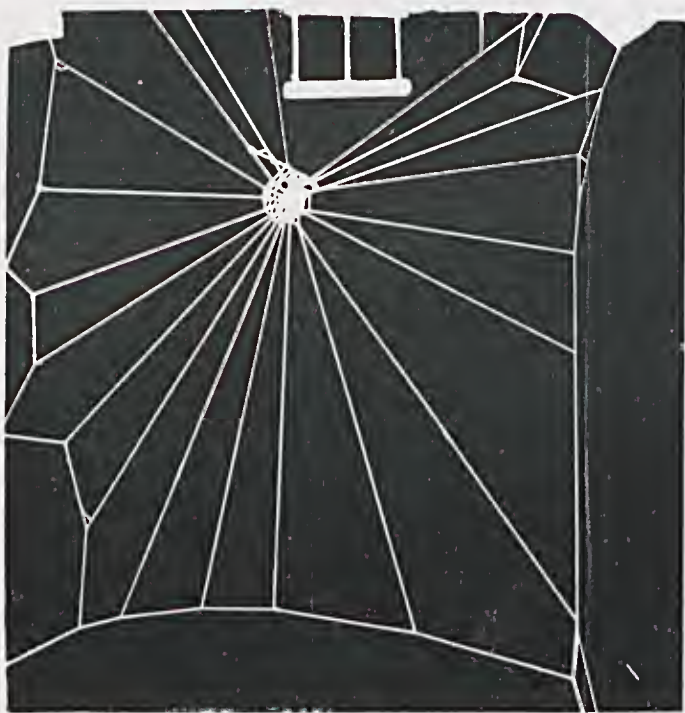



Fig. 2 — Radial and frame strueture of *Araneus* web. The spider has been removed before beginning provisional spiral from hub toward periphery. (From BREED, A. L., LEVINE, V. D., PEAKALL, D. B. and WITT, P. N., *Behaviour*, 23, 43, 1964).

The many parameters of the web may be reduced to three classes: the size, the shape, and the regularity of thread placement in the webs. Psychotropic drugs may be described in simply empirical terms of the effects they wreak on these classes. D-amphetamine, for example, in a dose of 600 mg/kg stimulates the building of smaller webs than normal with irregular placement of certain threads (the spiral and radial threads). LSD 25, on the other hand, can be administered in doses (0.3-0.5 mg/kg) which reduce the frequency of web-building, but have no effect on the regularity of angles and spirals; in fact the regularity of placement may be greater than usual. Higher doses do have an adverse effect on angle regularity.

These empirical observations, however, can only be the starting point for explanation. It is important of course to note that the webs change in ways specific to the drug, but it is necessary to identify basic elements in the execution of the web structure.

There are four sources of alteration which are now apparent.

Dr. Witt has discussed the possible role of *thread supply* in determining web dimensions. The inhibition or stimulation of protein-synthesis requires adjustments in the web. This is not an obvious relationship: it could be entertained as a possibility that the spider completes its web with thread material still available or that it runs short in the process. Ordinarily, however, the available material is rather completely utilized, the spider coming to a stop which not only completes the geometry of the web but empties the ampullate and aggregate glands. Diazepam, as Dr. Witt has shown, is an exception to this rule. An important point is that dearth or abundance of material need not imply loss of precision of placement of threads.

However, if cholinergic action enters into thread production, it must also be contemplated as one possible contributor to the second source of web change, *sensory-motor disturbance*. That is, neural transmission may be affected. Physostigmine, however, does not affect precision of placement although it does stimulate thread-synthesis. Two other substances potentially involved in neural transmission, on the other hand, nor-epinephrine and 5-hydroxytriptamine, affect both size and regularity of thread-placing. Whatever the source of the disturbance, it is clear that sensory-motor integration is a second factor underlying web alterations.

A related but conceptually independent source of disturbance may be called *failure or change in information-processing*. The capacity of the spider to integrate sensory information can be affected, for instance, by central nervous system lesions. A simpler form of interference is produced by depriving the spider of visual or tactual information by blinding or by removing legs.

Finally, it may be necessary to invoke a higher-order adaptation on the part of the animal, *restriction of energy output*. If a web is produced at all, it is smaller in area and in thread length; it may or may not show thread irregularities. Strychnine (300-820 mg/kg) for instance, diminishes frequency of building and results in webs of smaller spiral area and less oval shape. Caffeine (above 1 g/kg) also produces rounder webs, disruption in threads and long web-building times (several hours instead of the usual 20 to 30 minutes.)

While each or several of these factors may enter into the particular disturbances of web construction, we have recently been concentrating on the matter of information, chiefly on the sensory information employed by the spider in thread

Fig. 3 — Effect of d-amphetamine on web construction. The webs are separated by one day, but the spider has been given 600 mg/kg 12 hours before beginning the second web. Reduction in area and in number of radii are observable, but it can also be demonstrated that central angles and spiral separation are more irregular than usual. This figure and the following illustrate rather than establish drug effects; variation of webs requires statistical analysis of experimental treatment. (From WITT, P. N. BRETTSCHNEIDER, L., and BORIS, A. P., *J. Pharmacol. exp. Ther.*, **132**, 183, 1961).

Fig. 4 — Control, treatment and recovery webs, psilocybin. Drug was given 12 hours before web shown in B, dosage 150 mg/kg. Web A is web of previous day, C that of day following Web B. Reduction in area and number of radii in the treatment web are apparent. (From CHRISTIANSEN, A., BAUM, RICARDA, and WITT, P. N., *J. Pharmacol. exp. Ther.*, **136**, 31, 1962).

	A	*B*	*C*
Area (cm²)	293.6	227.3	314.5
Number of spiral turns	35	28	29
Number of radii	36	31	36

150 mg/kg psilocybin was given 12 hours before web B was built

placement. Both analysis of the completed webs and of ongoing building suggest that there are stages in construction in which testing functions alternate with actions taken upon those tests. A few examples will illustrate the observations.

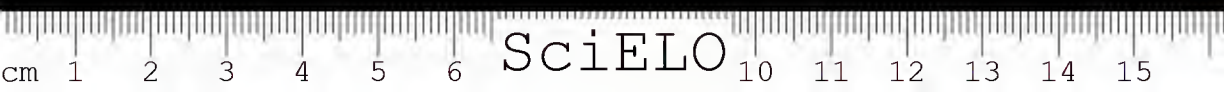
The first threads of the web are dependent upon the contingencies of location: upon the availability of fastening points. Even in the laboratory cages, the mooring threads differ from day to day, perhaps beginning from the apparently random residues of the previous day. One means or another will be employed to set the first threads; if an air stream from a fan is directed across the cage, the spider will let the thread be carried along it rather than laboriously carrying the dragline from place to place. But as construction proceeds, it is possible to speak of typical structures, where actions are guided by a narrower range of circumstances than in the initial stages and where it is possible to observe rather more invariable events. It is interesting that while the spider may be easily disturbed during the setting of the initial threads, it is considerably more difficult to do so once the later stages of construction have begun.

Assume that the spider has achieved a Y-structure of three threads with a sparse perimeter of additional framing threads. Taking a station at the center of this plexus or hub, the animal appears to test existing threads by circling, touching each of the lines with several legs; the necessity for an additional radial thread and/or supporting structures is apparently established during these movements, but it is not clear what sensory data are being utilized by the animal. One possibility is that feedback occurs from movement of a leg over a certain arc without contacting thread; another possibility is that the spider is utilizing some sensory data related to the degree of tension in the existing threads. At any rate, if the first legs on one side of *Araneus diadematus* are removed, there are clear and irreversible disturbances of the regularity of thread placement — although such an experiment does not allow us to distinguish between the two foregoing hypothesis.

Depending upon the results of the "test", the spider exits from the hub along an existing thread, pulling a dragline which is thereupon attached to the frame. The spider returns to the hub along the new radial thread. If the new placement is to occur in the lower quadrants of the web, the animal may drop by means of the dragline instead of crawling: there does seem to be an "option" and not a fixed action-pattern following the outcome of the probing function.

There is another option, a kind of substage which results not only in an additional radial line, but in an accessory supporting or framing member. Instead of returning directly to the hub along the new radial line the spider makes an attachment along the way. The attachment serves to secure the radial line a second time to the frame, and deviates it from its original direction. Both threads may receive additional reinforcement as the spider passes over them.

The assembling of the radial and frame elements proceeds by this alternation of testing and placement until the spider succeeds in making a complete circuit of the hub without receiving whatever signal it is that requires additional supports. The inauguration of the next stage is so imbedded in the preceding movements that it is disputable not only when it begins but whether it is a separate stage at all. The sequence of movements that have accompanied the radial-testing continue, but it is apparent that the spider has also been attaching thread to the radii as it circled, bridging the central angles. The result of a complete circuit of the web without need for a radial structure is the beginning of a tight spiral. Gradually the spider moves farther out from the center; the pro-

visional spiral has begun. This temporary spiral appears only as a trace in the completed web: it is devoured by the spider as it loses its usefulness as a guide and support for the laying of the permanent viscid spiral.

The permanent, tacky spiral is constructed in a clearly separable stage. First of all, the spider pauses at the completion of the provisional spiral. The aggregate glands now begin to supply material, and there seems to be a requirement of time for this glandular shift-over. (It is possible that the clear pause in the center of the web, which occurs in the radial building is also a period in which the gland duct is filling with material.)

The laying of the permanent spiral takes up by far the greater proportion of web-building time: the basic structures are build within the first 5 minutes, the permanent spiral occupies the remaining 15 to 20 minutes. The provisional spiral had proceeded from within outwards and has brought the spider to the periphery of the web; now the work goes from out inwards. Thread-placing is halted short of the hub, leaving an open space or free zone between the innermost turn and the hub.

The spider takes up its position of vigilance on the hub. It may remove some of the threads of the hub by biting them out.

Especially clear movie sequences of these and other activities of *Araneus* have recently been obtained by Barnum and Witt. These films have revived the speculation that the positioning of a particular thread is determined by the array of forces then extant in the web, that the spider tugs the threads and responds by laying a new thread if the resistance to the tug falls below a hypothetical but unknown value. In order to test this conjecture, we have been attempting to make films of the sequence of structures in a single web, with the objective of testing where the spider appears to test and predicting the position of the next thread. A successful emulation of the single event of new placement should permit a statement of the range of tension to which the spider is sensitive in building, if indeed this is the technological basis of its construction at all.

Discussion

A. Shulov: "Is there any correlation between the influence of drugs on the structure of the web and the changes in web's construction in time as a result of mutilation as cutting every one or two tarsi (Dr. Schleps experiments)?"

P. N. Witt and *C. F. Reed:* "The same structural change may be produced by different treatments. For instance, irregularities of central angles can be the consequence of losing one first leg or 600 mg/kg d-amphetamine."

W. Bücherl: "1. In what kind of spiders you made your studies? 2. Have you used also other species of spiders or only orb-web-spiders?"

P. N. Witt and *C. F. Reed:* "1. *Araneus diadematus* and *Zygiella-x-notata*. 2. The orb-weavers have the distinct advantage of producing webs with geometric forms susceptible to mathematical analysis. Departures from ordinary variation can therefore be specified in a way which is not possible with other webs."

D. Valente: "1. Há alguma correlação entre a feitura das teias e o sistema neuro-secretor? 2. O ciclo sexual e a idade têm influências na feitura das teias?"

P. N. Witt and *C. F. Reed:* "1. Endocrine structures have been histologically identified in spiders, but their function is still unknown. 2. All of our drug experiments were done with adult female *Araneus diadematus*, so that the drug would be the main variable. Males are irregular builders in later life."

67. THE EFFECT OF THE POISON OF SPIDER AND DIGGERWASPS ON THEIR PREY (*HYMENOPTERA: POMPILIDAE, SPHECIDAE*)

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Among the **HYMENOPTERA** we find the most sophisticated venomous animals. With regard to potency I do not know of a stronger poison than that of *Habrobracon juglandis*, a braconid wasp, which paralyzes its prey, the *Galleria* larva, even at a dilution of 1 part venom in 200,000,000 parts of hemolymph (1). Also with regard to duration of paralysis the predaceous wasps come first: a spider stung by the pompilid *Cryptochilus affinis* lived for four months in deep paralysis (2). One wonders why physiologists and pharmacologists have so far taken little interest in the solitary wasps.

I will confine myself in the following to the POMPILIDAE and SPHECIDAE. With their sting apparatus the females of these families inject venom into their prey which results in paralysis of varying duration. The pompilids hunt exclusively for spiders. On the list of prey of the SPHECIDAE we find all orders of insects and spiders too. Immobilization of the prey is always related to care of the offspring. An egg is laid on the victim and the hatching larva feeds on the living prey.

The main subject of this paper is the physiological effect of the poison. The duration of paralysis provides a first clue (3). When we look at the pompilids we find three categories of paralysis: 1. In the most primitive case represented by some members of the genus *Homonotus* the females paralyze spiders, lay an egg on the victim but bury them not in the ground. According to (4, 5) all the spiders recover a few minutes after the sting and resume their regular activity. The larva that hatches a few days later will feed on the spider and kill it. 2. Females of the genus *Anoplius* also subdue spiders by stinging. They bury them afterwards in chambers in the ground and attach an egg. If one digs them out one finds that paralysis lasts much longer than with *Homonotus*, nevertheless they usually recover after a few hours. In the chambers, however, they are, even after recovering, unable to move because they are packed so tightly. Temporary paralysis is also reported for *Notocyphus* (6). 3. Most of the pompilids paralyse the spiders deeply and permanently. The prey remains fresh, i.e. it is not desiccated or contaminated for weeks. Certainly the victims live during this time.

The striking fact is that the prey stung by certain pompilids is always able to recover, that of others is paralysed deeply and irreversibly. Since the stinging

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behavior is very similar in the whole family (7) one may assume that we have to deal with qualitative or quantitative differences in the composition of the poison or that different physiological systems are affected. Experiments on this point are much needed. Paralysis lasting only a few minutes could be caused by an instability of the active compound of the venom. The observed differences in duration of paralysis among members of the group that normally paralyse irreversibly can be attributed to the amount of poison injected as experiments with a sphecid wasp, reported later, show (8).

How and where the poison of pompilids acts in the organism of the prey is entirely unknown. There is one paper reporting that the venom gets into the hemolymph of the prey (9). Penetration of the sting into the central nervous system therefore seems not to be a prerequisite for paralysis and probably does not happen in the field.

To sum up, the venom of the POMPILIDAE has to be brought into the hemolymph of a spider and it paralyses the motor-system. There are interesting genus-specific differences in regard to duration and completeness of paralysis. Whether they are due to qualitative or functional differences of the injected venom still has to be established.

Now let us consider the SPHECIDAE. In this family we also find differences in duration and completeness of paralysis. Molecrickets stung by *Larra* recover always some minutes after stinging (10, 11). Crickets stored by *Sphex lobatus* also recover 10 to 15 minutes after being stung as well as the victims of some species of *Liris* and *Notogonia*. These examples, however, and the apparent killing of the prey by the venom of many BOMBICINI (12, 13), *Astata* species (14, 15) and some CRABRONINI are exceptional. Delay by many hours of onset of paralysis is reported for crickets stung by *Liris nigra* (16) and for ants hunted by *Aphilanthops frigidus* (17). The great bulk of the sphecoid wasps however paralyse their prey deeply and irreversibly.

I performed experiments with *Philanthus triangulum* (8, 18) which provided a first clue as to the effect of the poison of this species on its prey, the honeybee. In many cases I could watch the stinging directly. The bee is always stung only once into the cutaneous membrane behind the first pair of legs (Fig. 1). Histological sections demonstrated that the sting in most of the cases investigated did not pierce the ventral nerve cord. One can have bees stung by *Philanthus* experimentally at any desired point by cutting a small window into the cuticle and placing the sting there. It was shown that it is sufficient for paralysis that the venom merely gets into the hemolymph. The further the point of puncture is from the locomotor-system the longer it takes until the legs cease to move (Fig. 2). The natural point of puncture behind the first pair of legs guarantees the quickest onset of paralysis. Evidence was obtained as to the mechanism of immobilization. There are two facts indicating that paralysis is not due to a central action on the nervous system but rather to a peripheral blocking. The immobilization does not start instanstaneously by blocking the movements of all parts of the legs but spreads progressively toward the periphery. The distal parts of the legs can still move at a time when the proximal ones are already immobilized. Since all muscles of a given leg are innervated from the same main nerve trunk leaving the corresponding thoracic ganglion and since the cell bodies of the motoneurones lie closely together at the periphery of the ganglion, a central action of the venom should lead to a nearly simultaneous block or interruption of the efferent innervation of the various leg muscles. This

marked time delay can be explained by the anatomy of the insect leg. The muscles for movement of the coxa and femur lie in the body cavity of the bee and therefore will be reached by the venom first. The muscles for the tibia are attached in the leg itself, the ones for the metatarsus lie still further distally. It will take time until via the blood circulation enough poison has entered the narrow leg and therefore we encounter the delay in onset of paralysis.

Fig. 1 — Position of the diggerwasp *Philanthus triangulum* when attacking and stinging, behind the first pair of legs of its victim, the honeybee.

A second observation points in the same direction. With local administration of small doses of venom it is possible to paralyse muscles which are innervated by the very same ganglion as for example the musculature of the forewings without affecting the muscles of the middlelegs. Again one comes to the conclusion that this should be impossible by an effect of the venom on the CNS of the prey.

It was shown further for the venom of *Philanthus* that it acts not specifically on the honeybee but rather paralyses all tested insects and spiders too (Table I). The completeness of paralysis varies with the amount of poison injected. With little doses the bees can completely recover after initial im-
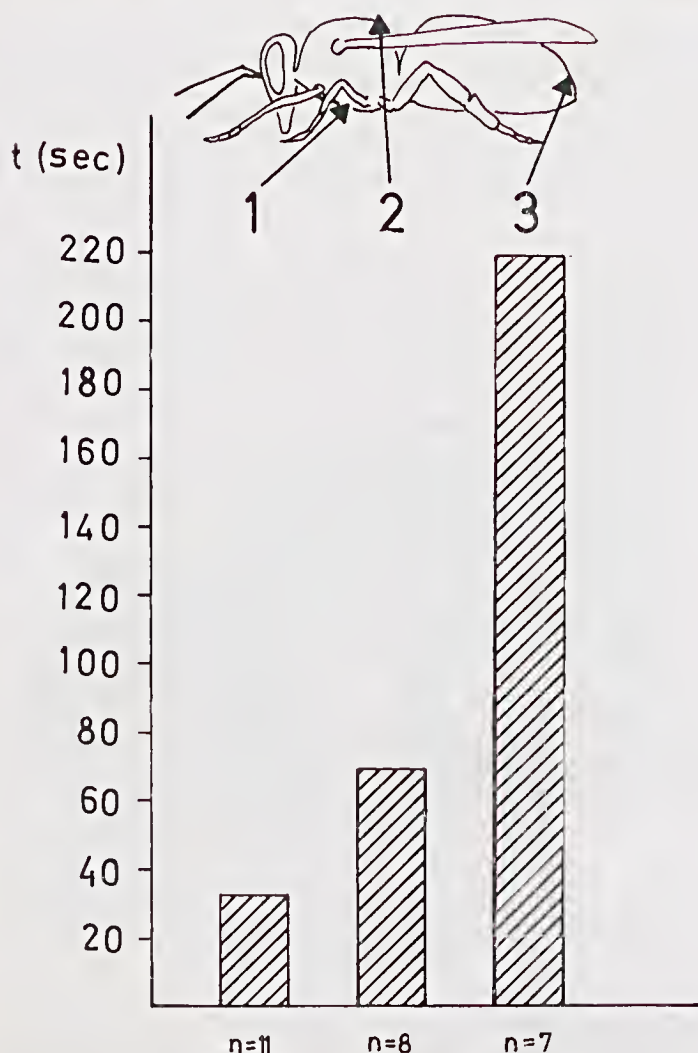
Fig. 2 — The variation of onset of paralysis of a honeybee's legs when venom of *Philanthus* was injected at points 1, 2 or 3.

mobilization. For a different species (19), demonstrated that duration and completeness of paralysis varied with the number of stings applied. I think in this case it is also an effect of the amount of venom injected.

Whether the result that the sting of *Philanthus* usually does not reach the CNS may be generalized to other species of sphecids requires histological studies of the paralysed prey. The precision with which *Liris nigra* applies four stings to the cricket (19) and the fact that they are placed in close topographical relation to the suboesophageal and the three thoracic ganglia leads to the assumption that in this case the venom has to be brought very close to the nervous centers responsible for locomotion. A variation of the mechanism of immobilization and also in the effect of the poison would not be surprising when one thinks of the great number of different insect families used as prey.

TABLE I — EFFECT OF INJECTED VENOM OF *PHILANTHUS TRIANGULUM* ON VARIOUS ARTHROPODS

ORDER	No. of families tested	No. of genera tested	No. of species tested	Effect
INSECTA:				
HYMENOPTERA	5	26	36	p
LEPIDOPTERA	2	4	4	p
COLEOPTERA	1	1	1	p
ORTHOPTERA	2	2	2	p
HEMIPTERA	2	2	2	p
ODONATA	1	1	1	p, d
DIPTERA	2	2	2	p, d
ARACHNIDA	1	1	1	p

p = paralysis, d = after initial paralysis apparently dead

As we have seen, recovery after initial paralysis is also possible in the SPHECIDAE. One cannot understand that in these cases either mechanical injury of the ventral nerve cord by the intruding sting or a heavy degeneration of the CNS as (20) claimed for caterpillars paralysed by *Ammophila* can happen. Most probably, blocking of the locomotor-system is also peripherally by spreading of the poison through the hemolymph to the effectors. This leads to a point of great physiological significance: is it the excitability of the muscle, the neuromuscular transmission at the junctions or the impulse propagation at the peripheral axons that are affected? In our laboratory experiments are in progress to study these questions in detail. Intracellular recording with microcapillaries from ventral body muscles of *Ephestia* larvae paralysed by *Bracon serinope* show that the membrane potential after 2 or 3 days is still between 43-65 mV compared with 42-71 mV of normal muscle fibers. The excitability of the musclefiber membrane therefore still exists. Mechanically stimulated, the musclefibers of paralysed larvae can contract. It is, however to early at the moment, to report more details on these experiments. Recently Piek (21) showed that the extracellularly recorded action potential of muscles of moth larvae declined and was finally abolished when venom of the braconid *Microbracon hebetor* was injected.

A few further observations should be mentioned which confirm the highly specific effect of the sphecid venom on the locomotor system. The heart muscles of the bee are unaffected by the venom of *Philanthus*. The heartbeats of a completely paralysed bee continues up to 37 hours. It is interesting to note that the frequency of beating is no longer regular but the heart beats in irregular bursts. Since it is generally assumed that the heart of adult insects is regulated by a neurogenic pacemaker it is tempting to assume that the venom affects this pacemaker and impairs neuromuscular transmission, not however the myogenic automatism.

The digestion of food in the gut is not stopped in paralysed caterpillars as the discharge of small fecal pellets, even several weeks after being stung by *Ammophila*, shows (22, Rathmayer unpublished). The web-spinning ability of paralysed spiders still is present (21) demonstrated that the metabolism measured as the oxygen consumption of paralysed insects does not differ much from that of a normal starving insect of the same species (Fig. 3).
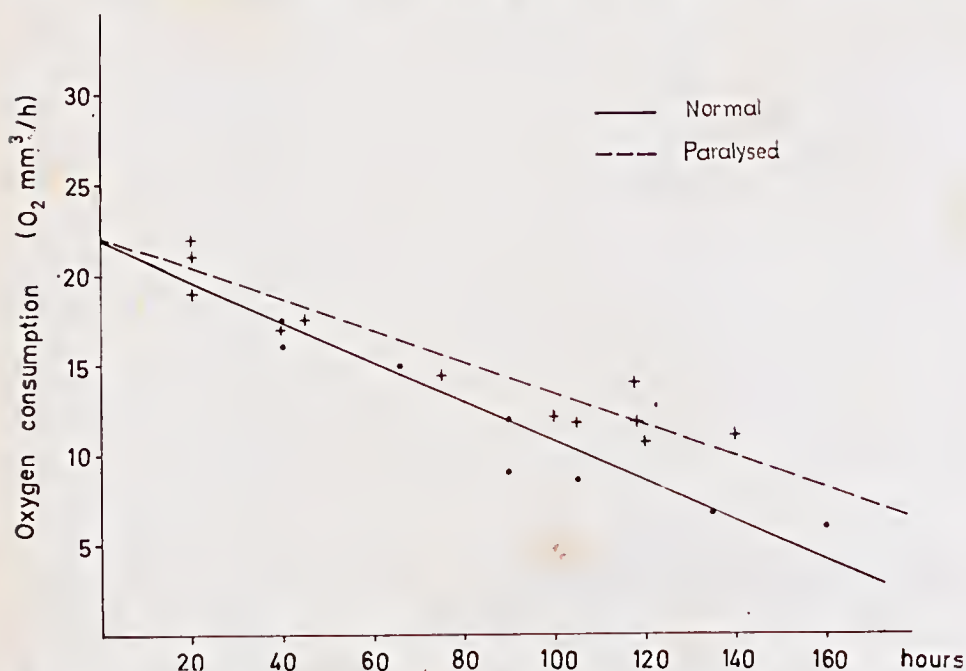
Fig. 3 — Oxygen consumption of caterpillars paralysed by *Ammophila campestris* compared with that of normal starving caterpillars (after NIELSEN, 1935).

With the assumption that we may draw a common conclusion from the experiments with *Philanthus* and the observations on the very few other species studied till now, the following point is reached. The venom of the sphecid wasps acts not on the CNS of the prey but peripherally by a selective inhibition of the locomotor-system. By the current electrophysiological experiments I hope to locate this peripheral site of action in the near future.

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